

Article

Should Tumor Infiltrating Lymphocytes, Androgen Receptor, and FOXA1 expression predict Clinical Outcome in Triple Negative Breast Cancer Patients?

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Abstract: Tumor-infiltrating lymphocytes (TILs) are a valuable indicator of the immune microenvironment that plays the central role in new anticancer drugs. TILs has a strong prognostic role in triple negative breast cancer (TNBC). Little is known about his interaction with Androgen receptor (AR) and Forkhead box A1 (FOXA1). We analyzed the relationships between TIL levels, AR and FOXA1 expression and their clinical significance in TNBC patients. Further, we investigated their interaction with other biomarkers like programmed cell death ligand-1 (PD-L1), Breast Cancer Type 1 susceptibility protein (BRCA1), Poly [ADP-Ribose] Polymerase 1 (PARP1) and Na⁺/H⁺ Exchanger Regulatory Factor 1 (NHERF1). The expression of the proteins was evaluated by immunohistochemistry in 124 TNBC samples. TILs were performed adhering to International TILs Working Group 2014 criteria. Cox proportional hazards models were also used to identify risk factors associated with poor prognosis. Multivariate analysis identified TILs as independent prognostic factor of disease free survival (DFS) ($p=0.045$). A Kaplan-Meier analysis revealed that the patients with high TILs had a better DFS compared to patients with low TILs ($p=0.037$), and the phenotypes TILs-/AR+ and TILs-/FOXA1- had a worse DFS ($p=0.032$, $p=0.001$ respectively). AR was associated with FOXA1 expression ($p=0.007$), and the tumors FOXA1+ presented low levels of TILs ($p=0.028$). A poor DFS was observed for AR+/FOXA1+ tumors compared to other TNBCs ($p=0.0117$). Low TILs score was associated with poor patients' survival, and TILs level in combination with AR or FOXA1 expression affected patient's clinical outcome. In addition, AR+/FOXA1+ phenotype identified a specific subgroup of TNBC patients with poor prognosis. These data may suggest new ways of therapeutic intervention to support current treatments.

Keywords: TILs; TNBC; AR; FOXA1; Prognosis

1. Introduction

Triple-negative breast cancer (TNBC) is characterized by lack of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (Her2) expression and/or amplification [1]. TNBC constitutes 15-20% of all breast cancers (BCs), having worse prognosis and a more aggressive clinical behavior respect to non-TNBCs [2-4]. Chemotherapy for

TNBCs remains still the standard treatment for the lack of specific molecular targets [5]. Therefore, there is an urgent need to identify new therapeutic approaches for the management of these patients. In the landscape of possible therapies new targets are emerging next to conventional treatment.

The importance of the immunologic control on cancers has long been recognized, as well as the immune-escape resulting from different factors. These remarks are true for all cancer types as much as for TNBCs [6,7]. Observations of tumor infiltrating lymphocytes (TILs) in cancer [8,9] and their association with favorable prognosis have changed the view about this disease [10]. Current studies give hope that a thorough knowledge of the interaction between tumor cells and the immune system might lead to clinically useful biomarkers. Among these are TILs and biomarkers related to the immune/tumor interaction, such as programmed cell death ligand-1 (PD-L1). These data underline the pivotal role of the immune system in cancer progression and therapy response, by supporting the use of tumor immune system biomarkers in clinical practice and the immunotherapy as a promising treatment strategy for this subtype of patients.

In addition, to immune checkpoint markers, whose role has become increasingly important, other biomarkers have assumed considerable significance, such as Androgen Receptor (AR) and Forkhead box A1 (FOXA1). In TNBC subgroup the co-expression of AR and FOXA1 has a prognostic value [11]. AR modulates the transcription of different genes by DNA-binding-dependent and independent mechanisms [12], including immune response genes [13]. Its expression is reported both in BC and in specific in TNBC [14], but the clinical significance of AR is still an open question. Recently AR has been associated to cancer cell growth and survival in TNBC cell lines and chemio-resistance in breast cancer models in vitro and in vivo [15-17]. Further, it is well-known that AR regulates transcriptional activity by different collaborative transcription factors, including FOXA1 [18] (Figure 1). FOXA1 has been identified as a transcriptional regulator of some liver-specific genes. It is expressed in different tumors, including BC and can bind to the promoters of more than hundred genes associated with regulation of cell signaling and the cell cycle, including ER. However, its role is unclear, some studies reported that FOXA1 and ER α constitute a major proliferative and survival axis for BC [19-22]. A recent research found that Breast Cancer Type 1 susceptibility protein (BRCA1) lack was related with the suppression of FOXA1 expression in BC cell lines and that BRCA1 mutation was linked to FOXA1 promoter methylation and silencing in BCs [23]. BRCA1 is a suppressor gene, whose dysfunction is linked to a higher risk of developing cancer, such as inhibition of DNA repair enzymes Poly [ADP-Ribose] Polymerase 1 (PARP1) [24]. Moreover, our team has shown in TNBC tumors that the association between nuclear PARP1 and cytoplasmic NHERF1 (Na⁺/H⁺ Exchanger Regulatory Factor 1) expression, a scaffolding protein with oncogenic activity [25], identified a subgroup of patients with a shorter survival [26].

In this study, we explored the significance of TILs, AR, FOXA1 expression and their impact on the clinical outcome of primary TNBC patients. Furthermore, we investigated their correlation with immunological (PD-L1), DNA repair (BRCA1, PARP1) and progression (NHERF1) biomarkers expression

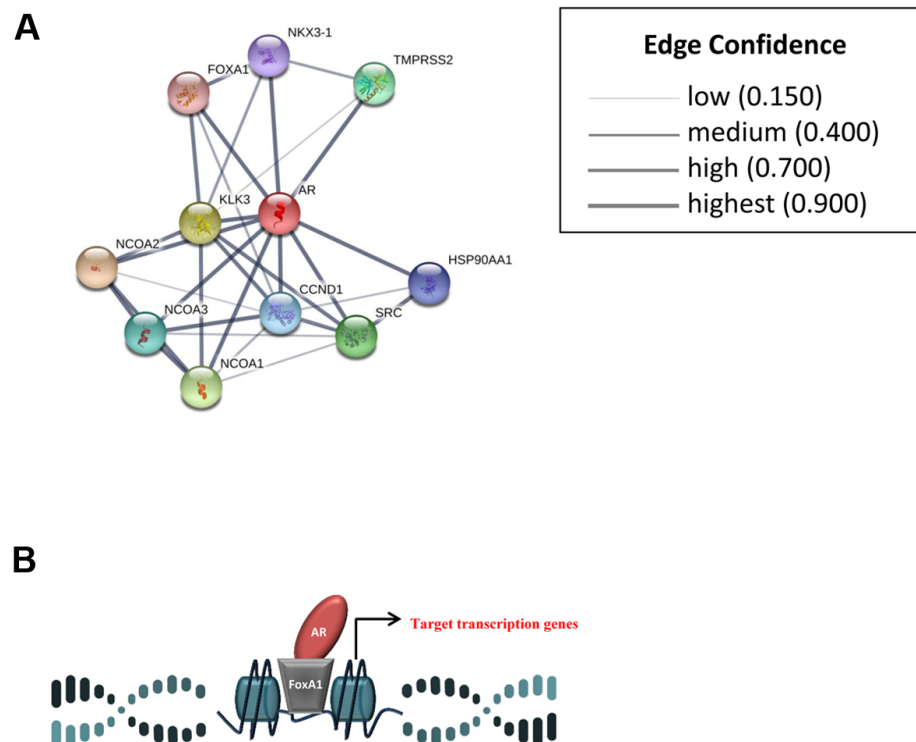


Figure 1. Scheme of Androgen Receptor (AR) transcriptional activity. **(A)** The scheme shows AR and its main interactors. High confidence protein-protein interaction network is generated with STRING software. The network nodes are input proteins. The edges represent the predicted associations. **(B)** in detail the interaction AR- Forkhead box A1 (FOXA1) is represented: FOXA1 should direct AR to sites usually occupied by ER, leading to an increase of proliferation estrogen-like.

2. Results

2.1. Protein Expression Profiling of AR, FOXA1, PD-L1, BRCA1, PARP1 and NHERF1

The expression of AR, FOXA1, PD-L1, BRCA1, PARP1 and NHERF1 was evaluated according to their specific cut-off as described in the Material and Methods section.

AR and FOXA1 expression was evaluated at nuclear level in the whole cohort. Among the stained BC samples, AR was present in 87% (108/124) of tumors and the 14.8% (16/108) of these tumors were AR+. The RNAscope assay confirmed the immunohistochemistry data, showing AR mRNA expression in the same tumor samples (Figure 2A).

FOXA1 was present in 91.1% (113/124) of tumor samples and it was overexpressed in 32.7% (37/113) of them. The PD-L1 reaction remained confined to the cell membrane. In 90.3% of the tumors (112/124) PD-L1 expressing the percentage of PD-L1 positive tumor cells was 25.9% (29/112). BRCA1 expression was assessed in 106/124 (85%) of the tumors and it was positive in 60/106 (56.6%) of cases. PARP1 expression was assessed in 107/124 (86.3%) of tumor samples, and it was positive in 20/107 (18.7%) of the tumors. NHERF1 expression was detected in the apical membrane, cytoplasm, and nucleus of tumor cells. These different localizations were scored separately and their significance was evaluated. Positive mNHERF1 was present in 35.6% (42/118), cNHERF1 in 52% (61/118) and nNHERF1 in 17% (20/118) of cases.

Some examples of FOXA1, PD-L1, BRCA1, PARP1 and NHERF1 staining patterns and tissue samples with low and high TILs presence are shown in Figure 2B and 2C respectively.

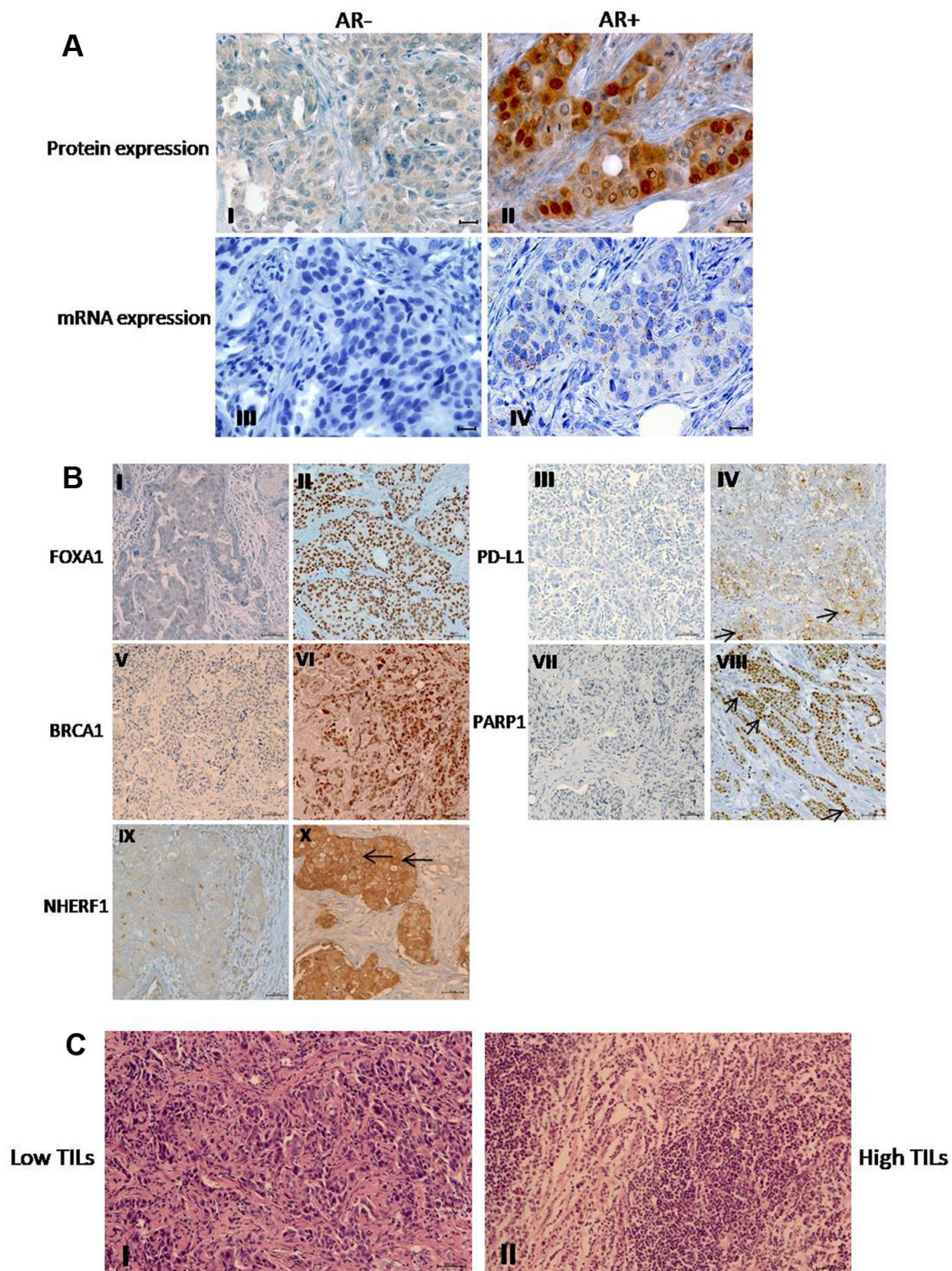


Figure 2. Immunohistochemical expression. (A) Androgen Receptor (AR) I) negative and II) positive immunohistochemical protein staining and the corresponding III) negative and IV) positive mRNA expression detected by RNAscope. Scale bars = 20 μ m. (B) representative images of immunohistochemical staining for Forkhead box A1 (FOXA1), programmed cell death ligand-1 (PD-L1), Breast Cancer Type 1 susceptibility

protein (BRCA1), Poly [ADP-Ribose] Polymerase 1 (PARP1) and Na⁺/H⁺ Exchanger Regulatory Factor 1 (NHERF1) proteins. **I**) negative and **II**) positive nuclear FOXA1 expression. **III**) negative and **IV**) positive PD-L1 expression, the positivity has been considered for tumor cells showing partial or complete membrane staining at any intensity (Arrows). **V**) negative and **VI**) positive high nuclear BRCA1 expression. **VII**) negative and **VIII**) positive nuclear PARP1 expression (Arrows). **IX**) negative and **X**) positive high membranous and cytoplasmic NHERF1 expression (Arrows). Scale bars = 50 μ m. **(C)** A representative tissue samples with **I**) low Tumor-infiltrating lymphocytes (TILs) and **II**) high TILs density. TILs was performed in full-face Hematoxylin & Eosin-stained sections. Scale bars = 50 μ m. Images were obtained on a Axion Image 2 upright microscope (Zeiss, Oberkochen, Germany) with a Axiocam 512 color camera.

2.2 Relationship between TumorMarkers Expression and Clinicopathological Features

A summary of significant associations between tumor marker expressions and clinicopathological features is listed in Table S1. Negative FOXA1 expression showed a significant association with higher tumor histological grade ($p=0.016$) in 88.2% of tumors. In addition, negative PD-L1 was observed in 95.2% of invasive ductal carcinomas ($p=0.048$) and all positive PD-L1 cases were significantly associated with high proliferative activity ($p=0.041$). High nNHERF1 expression was present in 75% of older patients ($p=0.004$), while the lack of nNHERF1 was noticeably associated with pre-menopausal status ($p=0.004$) in 73.5% of cases.

2.3 Association between Protein Expressions Analyzed

We also analyzed the association among the tumor biomarkers. The statistical analyses, using continuous variables, showed a direct significant association between AR and FOXA1 ($\tau = 0.354$; $p<0.001$) and cNHERF1 expression ($\tau = 0.241$; $p=0.002$). BRCA1 was directly correlated to PARP1 expression ($\tau = 0.248$; $p=0.001$), and PARP1 was directly linked to nNHERF1 ($\tau = 0.297$; $p<0.001$) and inversely to mNHERF1 expression ($\tau = -0.242$; $p=0.003$). When we analyzed TILs correlation, there was a direct relation comparing TILs with PD-L1 and cNHERF1 expression ($\tau = 0.259$; $p=0.001$; $\tau = 0.199$; $p=0.004$ respectively) and inverse relation between TILs and BRCA1 expression ($\tau = -0.172$; $p=0.020$). Statistical analysis and its relative heatmap are shown in Figure 3A and 3B. These results were also confirmed by dichotomized variables (data not show).

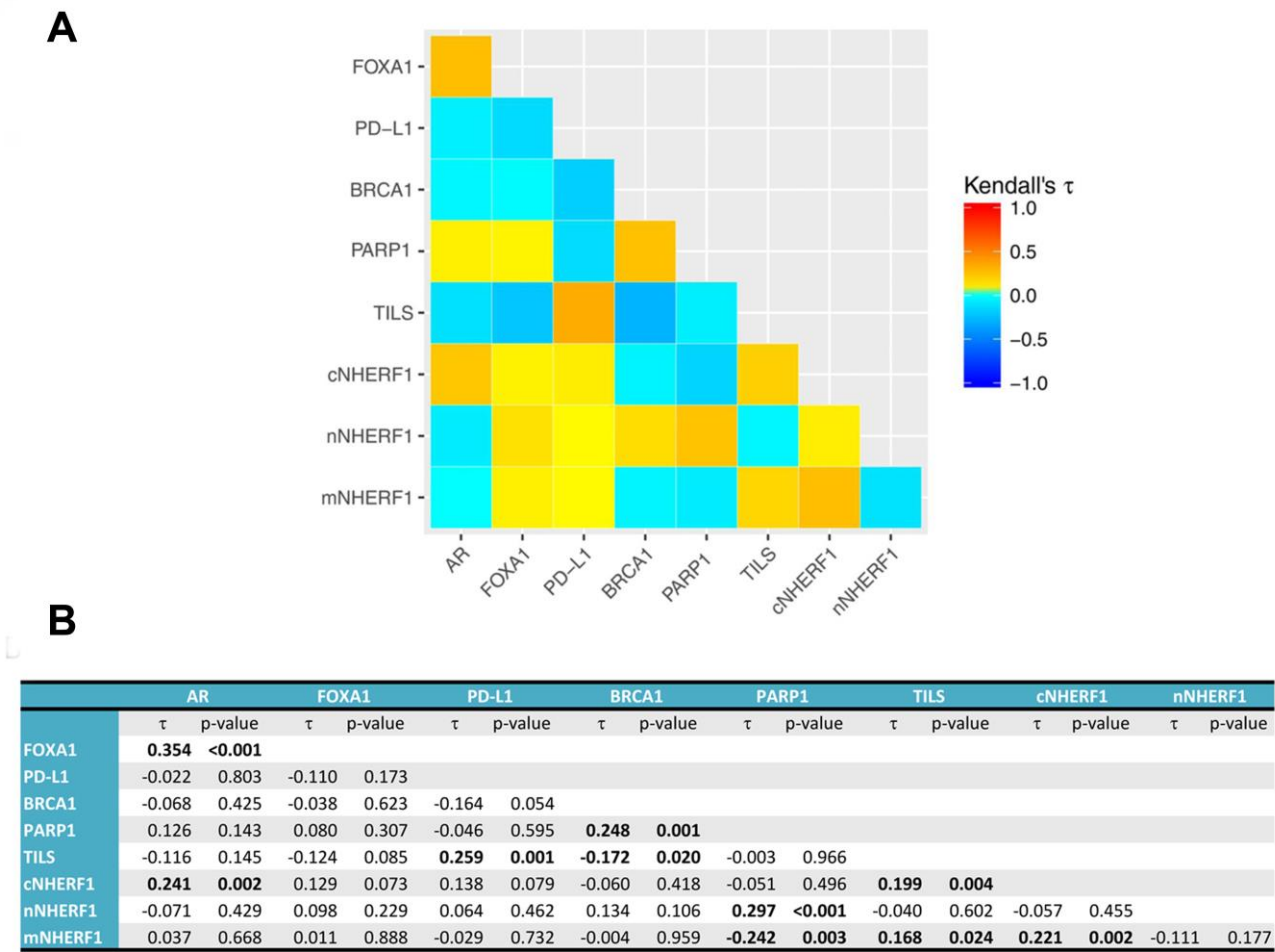


Figure 3. Protein interaction. **(A)** Heatmap of the protein-protein interaction. **(B)** The presence of non-linear relationship between two continuous variables was assessed by the Kendall rank test. AR: Androgen Receptor; FOXA1: Forkhead box A1; PD-L1: programmed cell death ligand-1; BRCA1: breast cancer susceptibility protein 1; PARP1: Poly [ADP-Ribose] Polymerase 1; Tumor-infiltrating lymphocytes (TILs); mNHERF1: membranous Na⁺/H⁺ Exchanger Regulatory Factor 1; cNHERF1: cytoplasmic NHERF1; nNHERF1: nuclear NHERF1.

2.4 Expression of Proteins and Patient Clinical Outcome

Univariate analyses were carried out for all the clinicopathological characteristics and the expression of AR, FOXA1, PD-L1, BRCA1, PARP1, mNHERF1, cNHERF1 and nNHERF1 proteins, as dichotomized and continuous variables. These were correlated to Disease free survival (DFS) and Overall survival (OS). Considering dichotomized variables, no significant differences were observed in the DFS and in the OS analyses among patients with high and low AR, FOXA1, PDL1 and NHERF1 protein expression. We found a significant association between BRCA1 and PARP1 with OS ($p=0.030$, $p=0.032$, respectively). Moreover, the subgroup of patients with high TILs had a better 5-year DFS and OS compared to patients with low TILs, 84% vs 75% ($p=0.037$) and 98% vs 88% ($p=0.019$) respectively (Table 1). Multivariate analysis identified the TILs as an independent prognostic factor of DFS (HR=0.34, 95% confidence interval (CI): 0.12–0.98, $p=0.045$, (Table 2).

Table 1. Univariate analysis on categorical data

Characteristics	pts.	DFS				OS		
		events	5-year DFS	HR	<i>p-value</i> ^c	events	5-year OS	<i>p-value</i> ^c
			(95% CI) ^a	(95% CI) ^b			(95% CI) ^a	
<i>Overall</i>	124	36	80 (73, 88)			12	92 (87, 97)	
<i>Age</i>								
≤ 51	65	22	79 (70, 90)	1.00	0.432	5	93 (87, 100)	0.460
> 51	59	14	81 (71, 92)	0.765 (0.39, 1.50)		7	89 (82, 98)	
<i>Pre/post</i>								
Post	73	25	81 (73, 90)	1.00	0.810	7	94 (88, 99)	0.391
Pre	41	11	79 (66, 93)	1.09 (0.53, 2.23)		5	87 (77, 98)	
<i>Size</i>								
≤ 2 cm	59	15	87 (78, 96)	1.00	0.552	4	93 (86, 100)	0.431
> 2 cm	64	21	74 (64, 86)	1.22 (0.63, 2.38)		8	90 (83, 98)	
<i>Hystological type</i>								
IDC	111	33	81 (74, 89)	-	0.947	9	93 (89, 98)	0.027
ILC	4	1	75 (43, 100)	-		0	100 (100, 100)	
Other	9	2	74 (48, 100)	-		3	67 (42, 100)	
<i>Lymph node status</i>								
Negative	65	16	83 (74, 93)	1.00	0.578	6	90 (83, 98)	0.894
Positive	55	17	79 (69, 91)	1.21 (0.61, 2.40)		6	92 (85, 100)	
<i>Ki67</i>								
Negative (≤ 20%)	12	4	82 (62, 100)	1.00	0.843	1	100 (100, 100)	0.945
Positive (> 20%)	110	30	81 (73, 89)	0.90 (0.32, 2.56)		11	90 (85, 96)	
<i>AR</i>								
Negative (< 10%)	92	23	83 (75, 91)	1.00	0.219	7	93 (88, 99)	0.111
Positive (≥ 10%)	16	6	81 (64, 100)	1.75 (0.70, 4.33)		3	87 (71, 100)	
<i>FOXA1</i>								
Negative	76	24	79 (71, 89)	1.00	0.281	8	90 (84, 97)	0.990
Positive	37	7	88 (77, 100)	0.63 (0.27, 1.47)		4	91 (82, 100)	
<i>PD-L1</i>								
Negative (< 1%)	83	24	83 (75, 92)	1.00	0.460	7	94 (88, 99)	0.432
Positive (≥ 1%)	22	7	79 (66, 96)	0.73 (0.31, 1.69)		4	86 (75, 100)	

BRCA1								
Negative (< 3%)	46	11	84 (74, 96)	1.00	0.273	1	100 (100, 100)	0.030
Positive (≥ 3%)	60	18	82 (72, 93)	1.52 (0.72, 3.23)		8	88 (80, 97)	
PARP1								
Negative (0-9)	87	24	83 (75, 91)	1.00	0.523	6	95 (91, 100)	0.032
Positive (10-18)	20	6	85 (71, 100)	1.34 (0.55, 3.28)		4	80 (63, 100)	
TILs								
Negative (< 50%)	70	26	75 (65, 87)	1.00	0.037	10	88 (80, 96)	0.019
Positive (≥ 50%)	47	10	84 (75, 96)	0.47 (0.23, 0.971)		1	98 (94, 100)	
mNHERF1								
Negative (0%)	76	19	81 (73, 91)	1.00	0.478	8	89 (82, 96)	0.435
Positive (> 0%)	42	14	78 (66, 92)	1.28 (0.64, 2.57)		3	95 (88, 100)	
cNHERF1								
Negative (70%)	57	14	81 (71, 93)	1.00	0.645	7	87 (78, 96)	0.202
Positive (≥ 70%)	61	19	79 (69, 90)	1.18 (0.59, 2.35)		4	95 (89, 100)	
nNHERF1								
Negative (0%)	98	17	78 (70, 87)	1.00	0.276	8	93 (87, 98)	0.321
Positive (> 0%)	20	3	89 (76, 100)	0.52 (0.16, 1.71)		3	83 (68, 100)	

^a five-year disease-free survival (DFS) or overall survival (OS) based on Kaplan-Meier method, b hazard-ratio (HR) computed using the Cox proportional hazard regression model for the DFS (for OS cannot be computed due to the low number of events), c p-value of the log-rank test for the equality of probability of an event (relapse for DFS or death for OS).); IDC: Invasive Ductal Carcinoma; ILC: Invasive Lobular Carcinoma; AR: Androgen Receptor; FOXA1: Forkhead box A1; PD-L1: programmed cell death ligand-1; BRCA1: breast cancer susceptibility protein 1; PARP1: Poly [ADP-Ribose] Polymerase 1; Tumor-infiltrating lymphocytes (TILs); mNHERF1: membranous Na⁺/H⁺ Exchanger Regulatory Factor 1; cNHERF1: cytoplasmic NHERF1; nNHERF1: nuclear NHERF1.

Table 2. DFS multivariate analysis on categorical and continuous variables

Characteristics	categorical		continuous	
	HR	<i>p</i> -value ^a	HR	<i>p</i> -value ^a
	(95% CI) ^a		(95% CI) ^a	
AR	1.65 (0.54, 5.02)	0.382	1.00 (0.99, 1.03)	0.509
FOXA1	0.38 (0.12, 1.20)	0.100	0.64 (0.16, 2.48)	0.517
PD-L1	0.49 (0.13, 1.76)	0.272	1.00 (0.96, 1.04)	0.863
BRCA1	1.47 (0.53, 4.11)	0.459	1.00 (0.99, 1.02)	0.386
PARP1	1.40 (0.46, 4.32)	0.554	1.00 (0.99, 1.02)	0.583
TILs	0.34 (0.12, 0.98)	0.045	0.19 (0.02, 1.51)	0.116
mNHERF1	2.28 (0.89, 5.82)	0.085	1.01 (1.00, 1.02)	0.104
cNHERF1	0.92 (0.37, 2.32)	0.867	1.00 (0.99, 1.02)	0.805
nNHERF1	0.62 (0.13, 3.00)	0.548	0.88 (0.70, 1.10)	0.263

^a hazard-ratio (HR) and *p*-value for five-year disease-free survival (DFS) computed using the multivariate Cox proportional hazard regression model with categorical and continuous variables. AR: Androgen Receptor; FOXA1: Forkhead box A1; PD-L1: programmed cell death ligand-1; BRCA1: breast cancer susceptibility protein 1; PARP1: Poly [ADP-Ribose] Polymerase 1; Tumor-infiltrating lymphocytes (TILs); mNHERF1: membranous Na⁺/H⁺ Exchanger Regulatory Factor 1; cNHERF1: cytoplasmic NHERF1; nNHERF1: nuclear NHERF1.

Next, we investigated the relationship between protein expression and TNBC patient’s survival. Kaplan-Meier curves revealed that the patients with higher TILs score had a better DFS (*p*=0.037), (Figure 4A).

Considering co-expression markers:

TILs-/AR-, TILs-/AR+, TILs+/AR-, TILs+/AR+, Kaplan-Meier analysis showed that the patients with TILs-/AR+ tumors had a worse DFS respect to the other phenotypes considered (*p*=0.032) (Figure 4B).

TILs-/FOXA1-, TILs-/FOXA1+, TILs+/FOXA1-, TILs+/FOXA1+ phenotypes, we observed significant differences in DFS among four groups. The subgroup TILs-/FOXA1-, had a worse DFS (*p*=0.001), compared to other groups (Figure 4c).

Moreover, a poor DFS was observed for AR+/FOXA1+ tumors respect to other tumors (*p*=0.0117) (Figure S1). The patients with AR+ and FOXA1- tumors tended toward a poorer DFS than patients with AR-/FOXA1+ tumors (*p*=0.080). The subgroup TILs-/BRCA1+ showed a worst DFS respect to TILs+/BRCA1- (*p*=0.097). Moreover, no significance was found considering the TILs+/PD-L1+ phenotype (*p*=0.100) (Figure S2).

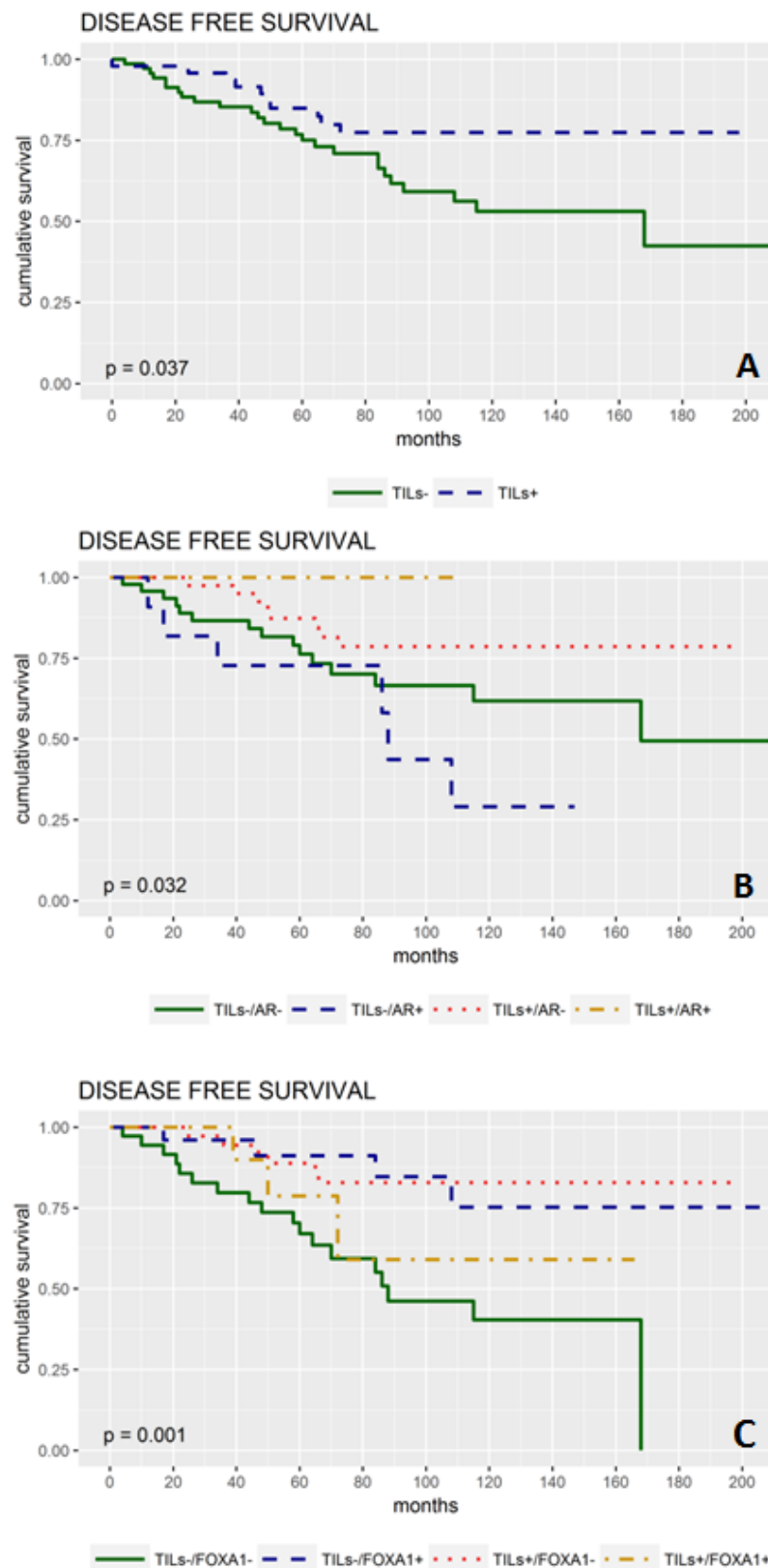


Figure 4. Survival analyses. **(A)** Disease-free-survival (DFS) curves for patients with Tumor-infiltrating lymphocytes positive (TILs+) versus TILs- presence ($p=0.037$). **(B)** DFS curves for patients with simultaneously TILs-/Androgen Receptor (AR)-, TILs-/AR+, TILs+/AR-, TILs+/AR+ expression: the TILs-/AR+ phenotype showed the worse DFS respect to the other phenotypes considered ($p=0.032$). **(C)** DFS curves for patients with

simultaneously TILs-/ Forkhead box A1 (FOXA1)-, TILs-/FOXA1+, TILs+/FOXA1-, TILs+/FOXA1+ expression: the TILs-/FOXA1- phenotype had the worse DFS compared to other groups ($p=0.001$).

3. Discussion

The prognostic relevance and the potential predictive impact of TILs in TNBCs has been recognized. Different studies confirmed that high levels of TILs are associated with better survival [1127-29]. In our series, multivariate analysis revealed that TILs is an independent prognostic factor of DFS, and, Kaplan-Meier analysis showed that TNBC patients with high TIL levels had a better DFS. In addition, when TILs are considered in association with AR, Kaplan-Meier analysis revealed a worse DFS for the TILs-/AR+ than other phenotypes. This result reinforces the key role that AR plays in more aggressive tumors and endorse the protective action of TILs. Patients with TILs-/FOXA1- tumors had a shorter prognosis than other subgroups, suggesting a pivotal role of the immune infiltrate. However a meta-analysis study also showed that low FOXA1 expression level was associated with a poor survival outcome [30].

Our results showed AR expression in about the 15% of cases, with a cut-offs of 10%, unlike Guin [11]. The choice of better cut-off is still subject of comparison. We adopted a cut-off of 10% following careful review of the literature [31, 32]. In agreement with previous studies, we observed a poor DFS in AR+ TNBCs, rationalizing a pharmacological AR block as a potential endocrine therapy for these patients [11,33]. The literature disagrees about the prognostic impact of AR in TNBC. In fact, some authors found an association of AR with a better prognosis in BC [33-36]. Heterogeneity of used antibodies, chosen cut-offs and patient cohorts make the AR expression particularly variable and its prognostic role controversial [37].

We underlined a significant association AR/FOXA1 and a shorter DFS in AR+/FOXA1+ phenotype, despite the small number of cases, in accordance with other authors [11]. Moreover, the involvement of AR in the tumor aggressiveness is also highlighted by the correlation between AR and cHERF1, a marker of tumor progression in BCs [25,38], and involved in different signaling pathway [39,40].

In this cohort of TNBCs there were not interesting data, among TILs ,AR, FOXA1 and DNA repair, immunological and progression biomarkers expression. An inverse relationship between TILs and BRCA1 has been found, hypothesizing that BRCA1-mutated tumors could have more tumor-specific neoantigens and, therefore, increased TILs. This is in line with what is reported by Massink, who observed that BRCA1-mutated BCs were affected by the presence of high numbers of TILs [41].

Some recent studies described an higher PD-L1 expression in TNBCs [11,42], but the rate of expression is extremely variable [43], and its prognostic significance is still debated. In the present study, about 26% of TNBCs expressed PD-L1 and it was associated to tumors with high proliferative activity, but no with AR no FOXA1. The DFS of patients with TILs+/PD-L1+ tumors was improved, as reported by other authors [44], even if our result was not statistically significant.

4. Materials and Methods

4.1 Patients and Clinicopathological Characteristics

A total of 124 primary TNBC patients who had received surgical treatment at the IRCCS Institute, Istituto Tumori "Giovanni Paolo II" of Bari, Italy between 1996 to 2012, were retrieved from a data base of the Pathology Department of our Institute. The patients were selected retrospectively according to the availability of the biological material and the clinical follow up. Our patient series was not consecutive. Characteristics of patients are shown in Table 3. All patients received adjuvant

chemotherapy (anthracycline and taxane). The patients were selected according to the following criteria: (i) female gender; (ii) histologically confirmed invasive carcinoma with estrogen (ER) and progesterone (PgR) receptor-negative tumors (<1% of positive cells), and any HER2 status (absence of HER2 overexpression or amplification), (iii) no evidence of distant metastasis at diagnosis, (iv) no any type of treatment prior to surgery, (v) no patients with history of previous malignancies. ER, PgR, proliferative activity and HER2 status were provided by the Pathology Department of our Institute. ER and PgR assessment used the ER/PgR PharmDX kits, Dako. HER2 status was evaluated using a monoclonal antibody (MoAb clone CB11; Novocastra Laboratories, Ltd., Newcastle, UK) and scored in accordance with the HercepTest scoring system (Food and Drug Administration) [45]. HER2 was considered to be positive if immunostaining was 3+ or if a score 2+ showed gene amplification by fluorescence in situ hybridization (FISH). Results were reported using ASCO/CAP 2007 criteria [46]. Ki67 nuclear staining was used to assess the proliferative activity, with a cut off value of 20% positive cells to indicate the tumors with Ki67>20% as highly proliferating. The analysis of TILs was assessed in full-face hematoxylin and eosin sections, according to the International TILs Working Group 2014 criteria [47]. Tumors with TILs score of $\geq 50\%$ were considered lymphocyte predominant breast cancer. The study was approved by the Ethics Committee of the Istituto Tumori "Giovanni Paolo II" with the reference 657/CE on 13th December 2018.

Table 3. Clinicopathological characteristics of 124 TNBC patients

Characteristics	n	(%)
<i>Patients Age: median value 51 (range 26-80)</i>		
≤ 51 years	65	(52.4)
> 51 years	59	(47.6)
<i>Menopausal status</i>		
Pre	83	(66.9)
Post	41	(33.1)
<i>Histological type</i>		
IDC	111	(89.5)
ILC	4	(3.2)
Other	9	(7.3)
<i>Histological grade</i>		
G1	1	(0.8)
G2	23	(18.7)
G3	99	(80.5)
Unknown	1	
<i>Tumor size (cm)</i>		
≤2 cm	59	(48.0)
>2 cm	64	(52.0)
Unknown	1	
<i>Lymph node status</i>		

Negative	65	(54.2)
Positive	55	(45.8)
Unknown	4	
<i>Ki67</i>		
Negative ($\leq 20\%$)	12	(9.8)
Positive ($> 20\%$)	110	(90.2)
Unknown	2	
<i>TILs</i>		
Negative ($<50\%$)	70	(59.8)
Positive ($\geq 50\%$)	47	(40.2)
Unknown	7	

IDC: Invasive Ductal Carcinoma; ILC: Invasive Lobular Carcinoma. Tumor-infiltrating lymphocytes positive (TILs).

4.2 TMA and Immunohistochemistry

Tissue microarrays (TMAs) were assembled from formalin-fixed and paraffin-embedded (FFPE) tissues of tumors using the Galileo Tissue MicroArrayer CK 4500 (Transgenomic). Each sample was arrayed in triplicate to minimize tissue loss and to overcome tumor heterogeneity.

Four-micrometer-thick sections were cut from FFPE blocks and mounted onto slides. The slides were processed and stained as previously reported [26].

The OptiView DAB IHC Detection Kit (Ventana Medical Systems) was used to detect protein expression and for PD-L1 staining an OptiView Amplification Kit was also used. All solutions were from Ventana Medical Systems unless otherwise specified. Briefly, slides underwent deparaffinization with the EZ PREP solution, followed by antigen retrieval with Cell Conditioning solution 1 at 95° for BRCA1 (60 min), AR (56 min), FoxA1 (32 min) and PD-L1 (32 min), and Cell Conditioning solution 2 at 95°C for PARP1 (36 min). No antigen retrieval was executed for NHERF1. The following step was incubation at 37° with the following specific primary antibody diluted in Antibody Diluent: rabbit polyclonal NHERF1 antibody (anti-EBP50; ThermoFisher Scientific, Rockford, IL, USA), 1:350 (16 min); mouse monoclonal PARP1 antibody (F-2 clone, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 1:500 (16 min); mouse monoclonal BRCA1 antibody (MS110 clone; Oncogene Research Products, Calbiochem, Merck KGaA, Darmstadt, Germany), 1:75 (32 min); mouse monoclonal anti-human PD-L1 (clone 22c3, Dako Agilent, Santa Clara, Ca, USA), 1:100 (32 min); mouse monoclonal anti-FoxA1 (clone 2f83, Merck Millipore, Burlington, Massachusetts, Stati Uniti), 1:200 (36 min); mouse monoclonal anti-human Androgen receptor (clone AR441, Dako agilent, Santa Clara, Ca, USA), 1:50 (36 min). Finally, tissues were counterstained with Haematoxylin and Bluing Reagent for 8 min and 4 min respectively, then were dehydrated and mounted. Positive and negative controls were included in each staining run as indicated in the data sheet of each antibody. All antibodies used in this study have been validated in the pre-analytic phase to guarantee a satisfactory level of reproducibility and accuracy.

4.3 Immunohistochemical Assessment

To analyze protein expression of AR, FoxA1, PD-L1, BRCA1, PARP1 and NHERF1 we utilized TMAs including 124 breast cancer samples. For immunohistochemical assessment we followed the previous method [26]. The data from IHC assay were examined independently by two of the researchers. If one core was uninformative, lost or contained no tumour tissue, the overall score applied was that of the remaining cores. For each analyzed protein, the cases in which all three cores were uninformative were considered non-assessable and excluded from the analyses. Any discrepancies between the two observers were resolved by re-examination and consensus. The examination of AR expression was assessed on the basis of nuclear staining intensity and we used 10% as cut-off value (negative <10; positive ≥ 10) [31].

For nuclear FoxA1 staining, the percentage of positive cells was estimated and the average intensity was scored similarly to PARP1. In this quickscore (QS) system a final score was calculated by multiplying the percentage score by the intensity score. The percentage of staining was categorized as: 0 = no nuclear expression; 1 = 1 to 10% positive tumor nuclei; 2 = 11 to 20%; and so on until a maximum score of 10 = 91 to 100% positive tumor nuclei. The intensity was scored as: 1+ = weak staining; 2+ = moderate staining; and 3+ = strong staining. A QS between 0 and 3 was classified as negative, and score ≥ 4 was considered positive [48]. PD-L1 protein expression was determined by using Tumor Proportion Score (TPS), which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity. A TPS $\geq 1\%$ was referred to as positive staining for PD-L1 [49]. The immunohistochemical assessment of BRCA1, PARP1 and NHERF1 was scored as previously reported [25, 50] and it was detailed in Table S2.

4.4 RNA Scope

For evaluation of AR mRNA expression a commercial kit (RNAscope® 2.5 High Definition (HD)-BROWN Assay, Advanced Cell Diagnostics, Newark, CA) has been used. Paraffin embedded samples were cut into 5 +/- 1 μm sections, baked for 1h at 60°C and deparaffinized. Subsequently the slides were pretreated with RNAscope® Pretreatment Kit (Hydrogen Peroxide, target retrieval solution and Protease Plus) to unmask target RNA and permeabilize cells. The probe was then hybridized to target mRNA for 2 h at 40°C. The signal was amplified using a multi-step process, followed by hybridization to horseradish peroxidase (HRP)-labeled probes. The signal was detected using a chromogenic substrate, 3,3'-Diaminobenzidine (DAB) for 10' at RT, and before mounting the slides were counterstained with 50% Hematoxylin I for 2' at RT and washed in 0,02% ammonia bath for 10 seconds. Tissue sections were examined under a standard bright field microscope at 20–40X magnification. Positive signals were visible as brown punctate dots.

4.5 Follow up and Statistical Analysis

The Chi-squared test and Kendall rank test were assessed to evaluate tumor markers relationship using categorical variables and continuous variables, respectively. Protein expression analyses was carried out in relation to disease-free survival (DFS) and overall survival (OS) in months. DFS was described as the time-frame between diagnosis and loco-regional/distant relapse (second invasive BC, second primary cancer and/or death without evidence of BC to the date of last contact). OS was described as the time-frame between diagnosis and date of last contact or of death from any cause. Univariate analyses of biomarkers expression and clinicopathological variables was performed for both DFS and OS, using Kaplan-Meier method [5-year, 95% confidence interval (CI)] and compared by the log rank test. Hazard-ratio (HR) for Univariate and Multivariate analyses was calculated by

the Cox proportional hazard regression model. Statistical significance level was p-values <0.05. Statistical analyses were achieved using the statistical packages survival and survminer of the statistical language R, version 3.4 [51].

5. Conclusions

Our data proved that low TILs score was associated with poor patients' survival, and the presence or absence of TILs in combination with AR or FOXA1 expression affected patient's clinical outcome. Moreover, AR+/FOXA1+ phenotype identified a specific subgroup of TNBC patients with a poor DFS. The better DFS of the TILs+/PD-L1+ tumors suggests a general activation of immune system in TNBCs, highlighted also by direct correlation between TILs and PD-L1. In this context, our results suggest that TILs may be a good marker of the immune response, and underline the need of future studies on the relationship between the immune system and cancer cells.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Relationship between tumor markers and clinicopathological features; Table S2: Dilution, source, staining of antibodies and cut off used; Figure S1: Survival analysis. DFS curves for patients with simultaneously AR+/FOXA1+ phenotype respect to all other tumors (p=0.0117); Figure S2: Survival analysis. DFS curves for patients with simultaneously AR-/FOXA1+ vs AR+/FOXA1- (p=0.080). TILs-/BRCA1+ vs to TILs+/BRCA1- (p=0.097) and TILs+/PD-L1+ Vs TILs-/PD-L1- (p=0.100) expression.

Author Contributions: A.M. and G.O. conceived and designed the study; M.C. and C.V. developed methodology; F.G. provided patients' follow-up; O.P. and M.I.P. provided clinical-pathological features; A.V. analyzed data; C.S. writing-original draft preparation; A.M. writing-review and editing; A.M. and N.S. supervision.

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