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Original Contribution

Correlation of folate receptor alpha expression with clinicopathological parameters and outcome in triple negative breast cancer

Irena Zagorac[a,b,*], Branka Lončar[b,c], Branko Dmitrović[a,b,d], Kristina Kralik[b], Andrej Kovačević[a,b]

[a] Department of Pathology and Forensic Medicine, Osijek University Hospital, Josipa Huttlera 4, HR-31000 Osijek, Croatia
[b] Faculty of Medicine, University of Osijek, Josipa Huttlera 4, HR-31000 Osijek, Croatia
[c] Department of Clinical Cytology, Osijek University Hospital, Josipa Huttlera 4, HR-31000 Osijek, Croatia
[d] Faculty of Dental Medicine and Health, University of Osijek, Crkvena 21, HR-31000 Osijek, Croatia

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ABSTRACT

Folate receptor alpha (FRα) is a membrane-bound protein with a high affinity for folate, which is necessary for the biosynthesis of amino acids and nucleotide bases. It has been shown to be a potential prognostic and therapeutic target, primarily in lung and ovarian cancer, as well as in breast cancer. The aim of this study was to examine FRα expression in a cohort of patients with triple negative breast cancer (TNBC), in correlation with clinicopathological parameters and prognostic factors. By using polyclonal FRα antibody on archival paraffin blocks immunohistochemistry was performed. To evaluate the expression of FRα, H-score was used, which marks both the proportion of stained cells and the intensity of staining. Statistical analysis correlating FRα expression with clinicopathologic parameters and clinical outcome were performed. FRα was expressed in most of the patients (85%). Significant correlation of expression and histologic grade (Mann Whitney *U* test, P = 0,03) and type of tumor (P = 0,02), was found. It was noticed that with higher Ki-67 proliferation index values, H-score has lower values (r = −0,284, P = 0,006). Multivariant regression analysis (Cox regression, *Stepwise* method) showed H-score as a significant predictor for the risk of disease recurrence (OR = 1,005, P = 0,04). No correlation between FRα expression and overall survival (OS) and disease-free survival (DFS) was found.

In conclusion, FRα is highly expressed in TNBC, and, given the correlation with clinicopathological parameters, subpopulation of patients could be identified that could be potential targets for new therapeutic perspectives in the treatment of this breast cancer subtype.

1. Introduction

Breast cancer is the most prevalent cancer in women with over 2 million newly diagnosed cases in 2018 and mortality of 15% in the same year (approx. 627,000 deaths) [1]. High incidence and, unfortunately, still high mortality, makes it an important global, healthcare and socioeconomic problem. Epidemiological studies have found several exogenous and endogenous factors that affect breast cancer occurrence, the most important ones being sex, age and genetic predisposition [2-6]. The disease itself is heterogeneous with the majority of breast cancers expressing estrogen (ER) and progesterone (PR) receptor, 10–15% overexpressing HER2, while approximately 15% are of the triple negative phenotype (TNBC).

TNBC is characterized by a lack of ER, PR, and HER2 expression, and, typically, is of greater dimensions, has a higher grade and more aggressive disease course with lower survival rates, when compared to other phenotypes [7-9]. Unlike hormone receptor positive, and HER2 positive breast cancers, which can be treated with targeted therapy, TNBC is currently treated with chemotherapy alone, and, as such, poses a great challenge for doctors as well as a great field of research, since new therapeutic approaches are needed for this poor-prognostic subtype [10-12].

Folate receptor alpha (FRα) is a membrane-bound protein with a high affinity for folate, which is necessary for the biosynthesis of amino acids, nucleotide bases, and other methylated compounds. Hence, the importance of folate during the growth of cancer cells is displayed in its essential role in the proliferation, growth, and survival of cells. FRα is located on the apical cell surface in a restricted number of healthy tissues, such as fallopian tubes, pneumocytes and kidney tubules [13-15]. However, overexpression of FRα has been found in various epithelial tumors, including the kidney, lungs, ovaries, uterus, bladder, colon and breast [13,14,16,17]. Limited distribution and expression in

* Corresponding author at: Department of Pathology and Forensic Medicine, Osijek University Hospital, Josipa Huttlera 4, HR-31000 Osijek, Croatia; Faculty of Medicine, University of Osijek, Josipa Huttlera 4, HR-31000 Osijek, Croatia.
E-mail address: irena.zagorac88@gmail.com (I. Zagorac).

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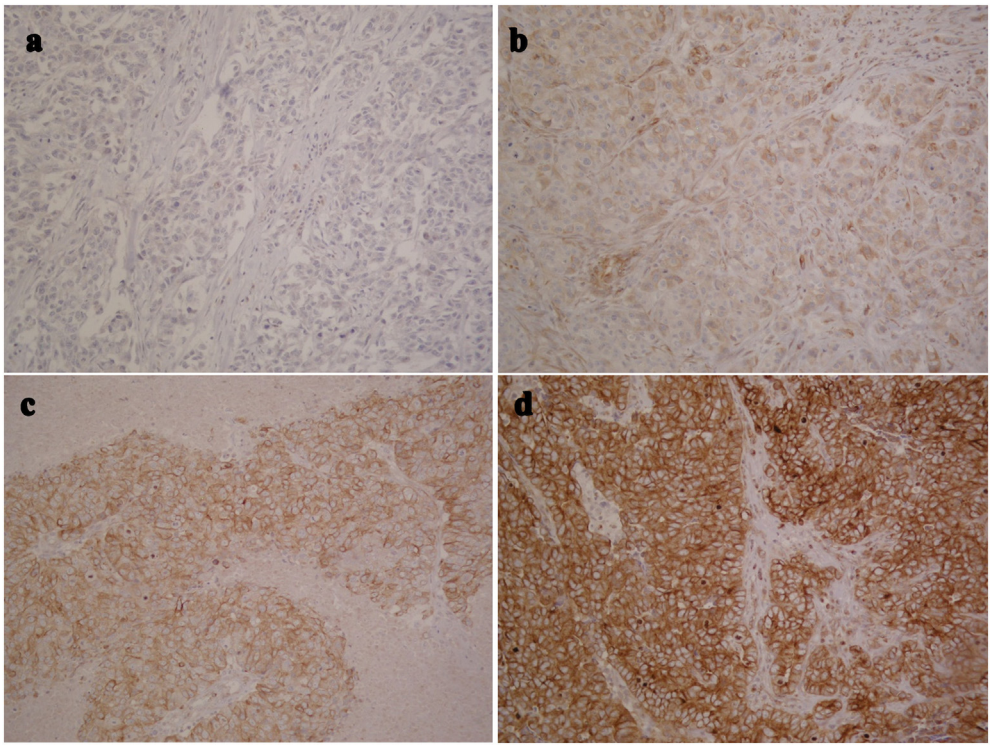

Fig. 1. Representative FRα expression in triple negative breast carcinoma. H-score was calculated, which marks both the proportion of stained cells and the intensity of staining. H-score was defined with formula: *H-score* = (% of cells staining with intensity 1+ × 1) + (% of cells staining with intensity 2+ × 2) + (% of cells staining with intensity 3+ × 3). a H-score = 0, b H-score = 50, c H-score = 140, d H-score = 280.

specific malignancies make this receptor potential target for tumor strategies and diagnosis, therefore it is important to identify patients who may have an advantage from FRα targeted therapies. Several studies have shown that FRα expression in breast cancer negatively correlates with ER and PR expression, and, when compared to other breast cancer subtypes, TNBC shows higher expression of FRα [18,19].

The aim of this study was to investigate the expression of FRα in a cohort of patients with TNBC, to determine its correlation with clinicopathological parameters and clinical outcome (disease-free survival (DFS) and overall survival (OS)).

2. Material and methods

2.1. Patient cohort

This retrospective study included 92 patients diagnosed with TNBC, who underwent surgical excision, in the period from January 1st, 2008 to December 31st, 2016, at the University Hospital Centre Osijek. Archival materials and pathology files used for evaluation were obtained from the Clinical Department of Pathology and Forensic medicine, University Hospital Centre Osijek and relevant clinical data were obtained from the Department of Oncology, University Hospital Centre Osijek. Factors of inclusion were primary TNBC, while factors of exclusion were the history of malignant breast cancer, other subtypes of breast cancer phenotype, unknown ER, PR or HER2 status and evidence of metastatic disease prior to the surgical excision of the tumor.

2.2. Immunohistochemistry method and scoring

Immunohistochemistry was performed with polyclonal rabbit antibody Folate Receptor alpha Polyclonal AntibodyC (ThermoFisher Scientific, Cat. No. PA5-42004) using Ventana BenchMark Ultra (Roche). Formalin fixed paraffin embedded tissue blocks were used to make 4 μm sections that were deparaffinized in xylene and then rinsed for 20 min with CC1 – Cell Conditioner (Tris/borate/buffer, pH – 8.4). Afterward, Universal DAB inhibitor was applied (3% H_2O_2), for 5 min at 37 °C, followed by 10-minute rinsing with reaction buffer (Tris buffer, pH – 7.6). Enzymatic digestion using HRP multimer, with incubation of 4 min at 37 °C, was done and another rinsing with reaction buffer, for 10 min at room temperature, was performed. After the rinse, the primary Folate receptor alpha antibody was applied (1:80 dilution, 60 min at 37 °C). After primary antibody, incubation with Ultra Conditioner was performed, followed by the application of Ultra View DAB Copper ($CuSO_4$), for 5 min at 37 °C. Following that, rinsing for 10 min at 37 °C and staining with hematoxylin for 12 min at room temperature were made. Another rinsing with reaction buffer at room temperature was done, for 5 min, and then impregnation with ULTRA LCS (Ultra liquid Coverslip). Rinsing of slides and dehydration with sequential ethanol baths, 70%–100% was performed. They were then bathed with xylene before coverslips on automated coverslipper (Sakura) were applied.

Slides were evaluated under a light microscope by two independent pathologists, both blinded to clinicopathologic information and clinical outcome. They were analyzed under 4×, 10×, 20× and 40× objectives. 3+ staining reflected thick membranous staining, already visible at 4× and confirmed at 10× objective. 2+ staining was thinner and weaker in intensity than 3+ staining, visible at 10× and confirmed at 20× objective, while 1+ staining requested 20× and/or 40× objective for interpretation, and reflected very thin, weak staining of membrane. For the FRα expression assessment, the H-score method, which marks both the proportion of stained cells and the intensity of staining, was used. H-score varied from 0 to 300 and, for each sample, was defined with formula: *H-score* = (% of cells staining with intensity 1+ × 1) + (% of cells staining with intensity 2+ × 2) + (% of cells staining with intensity 3+ × 3). For the DFS and OS analysis, for the purpose of this study, values of H-score < 10 were considered as negative folate receptor alpha expression, and positive values were split into 3 subgroups, as follows: low (H-score 10–100), moderate (101–200) and strong (201–300) expression. Representative images are shown in Fig. 1.

2.3. Statistical methods

Categorical data were presented as absolute and relative

frequencies. Numerical data were shown with median and interquartile range. Fisher's exact test was used to assess the differences in categorical data. Shapiro-Wilk test was used to examine the normality of numerical variables distribution. Numerical variable differences between two independent groups were analyzed by the Mann-Whitney *U* test.

The statistical association was measured by Pearson's correlation coefficient (r). Kaplan-Meier survival curves were compared by the log-rank test. Multivariate Cox regression analysis (*Stepwise* method) was used to predict the probability of disease recurrence and expressed with an odds ratio (OR) and 95% confidence interval (CI). The receiver operating curve (ROC) analysis was used to determine the optimal threshold, area under the curve (AUC), specificity, and sensitivity of the tested parameters. All P-values were two-sided.

The significance level was set at $\alpha = 0{,}05$. MedCalc Statistical Software version 18.2.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018) and IBM SPSS Statistics 23 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) was used for statistical analysis.

3. Results

3.1. Patient characteristics

Invasive breast cancer of triple negative phenotype from 92 women was studied in this analysis, with a median age of 58 (interquartile range: 49–68), the youngest patient being 31, and the oldest 89 years of age. The clinicopathologic characteristics of the tumors are listed in Table 1. Tumors were almost equally left (53%) and right (47%) sided. Only 2 (2%) of the studied patients had lobular histology while the rest of them (90/92; 98%) were ductal. Of the 92 women, the majority presented with grade III carcinoma at the time of diagnosis (71%), fewer were of grade II (28%), while only one patient presented with grade I carcinoma (1%). Lymphovascular invasion was present in only 20 patients (22%). 36 (39%) patients had node-positive disease, with most of them having 1–3 positive axillary lymph nodes (26%), a somewhat higher number of patients had the node-negative disease (52/92; 57%), and for four patients nodal status was unknown. 48% of women had T1 tumors (< 2 cm). The vast majority of patients (92%) had a Ki-67 proliferation index ≥20%. Disease relapse (whether in a form of local recurrence or distant metastases) was found in 21% of cases.

Table 1
Clinicopathological characteristics of tumors.

	No. (%)
Location	
Right breast	43 (47)
Left breast	49 (53)
Histology	
Invasive ductal carcinoma	90 (98)
Invasive lobular carcinoma	2 (2)
Histologic grade	
I	1 (1)
II	26 (28)
III	65 (71)
Lymphovascular invasion	
Present	20 (22)
Absent	72 (78)
Nodal status	
N+	36 (39)
N−	52 (57)
Unknown	4 (4)
Nodal status, nr. of +	
0	52 (57)
1–3	24 (26)
4–9	7 (8)
≥10	5 (5)
Unknown	4 (4)
Tumor size, cm	
≤0,5	1 (1)
> 0,5–≤1	7 (8)
> 1–≤2	36 (39)
> 2–≤5	38 (41)
> 5	9 (10)
Unknown	1 (1)
Ki-67	
< 20%	7 (8)
≥20%	85 (92)
Relapse	
Yes	19 (21)
No	73 (79)

N+, lymph node positive; N−, lymph node negative.

3.2. FRα expression in TNBC

The median of the H-score was 100 (interquartile range: 35–188,75). The correlation of H-score with clinicopathological characteristics of tumors is shown in Table 2. Significantly higher H-score was present in women with lobular cancer histology (Mann Whitney *U* test, P = 0,02). Regarding the tumor grade, significantly lower H-score values were found in patients with grade III carcinoma (Mann Whitney U test, P = 0,03), compared to the patients with grade II. Also, there was a statistically significant association between H-score and relapse occurrence, with somewhat higher H-score values in women that had a relapse (median 127,5) (interquartile range: 90–215; Mann Whitney U test, P = 0,04), than in those that had no relapse (median 85). No association of H-score with lymphovascular invasion, node status (+/−) and Ki-67 (< 20%/≥20%), has been found.

Pearson's coefficient of correlation (r) has been used to measure the correlation of H-score with age, number of positive lymph nodes, tumor size, and Ki-67 proliferation index. It was noticed that H-score is significantly correlated with Ki-67, that is, for higher values of Ki-67, H-score takes on lower values (r = −0,284 P = 0,006). Also, higher Ki-67 values were found in tumors of greater size (r = 0,210 P = 0,04).

Cox regression was used to investigate the effect of H-score upon the risk of disease recurrence. Using multivariate regression analysis (*Stepwise* method) H-score is a significant predictor for the risk of disease recurrence (OR = 1,005, P = 0,04).

To estimate the H-score value that regression analysis has shown to have a significant impact on the risk of disease recurrence, the ROC curve was used. The cut-off point was altered so that by creating a ROC curve, it could be objectively determined which value best distinguishes compared group. In our results, H-score can be shown as a diagnostic indicator of the risk of disease recurrence (Table 3, Fig. 2).

To analyze the relationship between FRα expression and survival, for the purpose of this study, H-score < 10 was considered as negative expression, while positive values were split into 3 subgroups: low (H-score 10–100), moderate (101–200) and strong (201–300) expression. Overall survival (OS) was determined as the time elapsed since the surgical excision of the tumor to the time of death or last follow-up, and disease-free survival (DFS) was determined as the time from the surgical excision of the tumor to the time of relapse (locoregional or distant recurrence). The arithmetic mean of OS was 64.7 months (95% CI 53, 1 to 76,2 months), without significant difference in overall survival regarding whether FRα is positive or negative (P = 0.51, log-rank test). Also, no association was found in DFS (arithmetic mean 108,3 months; 95% CI 94,03 to 122,05 months) when correlated with positive and negative FRα (P = 0.25, log-rank test) (Fig. 3A and B).

The expression of FRα was also analyzed for one-year and three-year survival periods. One-year survival was 80% and 3-year 58.9%, without statistical significance regarding the positive or negative expression of FRα (P = 0,86; P = 0,71 log-rank test) (Fig. 3C–F).

Table 2
Correlation of H-score with clinicopathological characteristics.

	H-score median (interquartile range)	Hodges-Lehmann difference	95% CI	P*
Histology				
Invasive ductal carcinoma	100 (35–180)	197,5	–	0,02**
Invasive lobular carcinoma	295 (290–300)			
Histologic grade				
I (n = 1)	290			
II	145 (80–215)	−50	−100–0	0,03**
III	90 (31,25–148,75)			
Lymphovascular invasion				
Present	62,5 (0−130)	35	0–85	0,09
Absent	112,5 (40–190)			
Nodal status				
N+	92,5 (15,5–192,5)	0	−35–45	0,86
N−	97,5 (37,5–165)			
Ki-67				
< 20%	200 (85–280)	−80	−165–0	0,06
≥20%	100 (31,25–176,25)			
Relapse				
No	85 (15,5–177,5)	55	0–95	0,04**
Yes	127,5 (90–215)			

CI, confidence interval; N+, lymph node positive; N−, lymph node negative.
* Mann Whitney *U* test.
** Statistically significant (P < 0.05).

Additionally, we analyzed survival regarding positive FRα values. The arithmetic mean of OS was 63 months (95% CI 50,7 to 75,2 months), without a statistically significant association regarding positive values of FRα expression (P = 0.64, log-rank test). Likewise, no statistically significant association was found between DFS (arithmetic mean 105 months; 95% CI 89,5 to 120,6 months) and positive values of FRα expression (P = 0.29, log-rank test) (Fig. 4A and B).

One-year survival was 80% and 3-year survival was 57.9%, without statistical significance regarding positive values of FRα expression (P = 0,24; P = 0,39, log-rank test) (Fig. 4C–F).

4. Discussion

Triple negative breast cancer is defined by negative expression of ER, PR, and HER2, and is, as such, insensitive to available hormonal and HER2 based therapies. It occurs in younger women and is characterized by high histologic grade, greater dimensions when compared to other subtypes, a high index of proliferation and a poor prognosis. Although it accounts for only 15% of all breast cancer subtypes, given the aforementioned characteristics, it is responsible for the high number of breast cancer mortality. Therapy of that breast cancer phenotype currently includes highly toxic and unspecific chemotherapeutic protocols, which, with all stated, indicates the need of finding new biomarkers to make progress in the development of therapeutic agents directed in treatment of this clinically aggressive phenotype [7,20].

A biomarker that is currently being researched is folate receptor alpha, a glycosylphosphatidylinositol anchored membrane protein that binds folic acid with high affinity [22,23]. Studies have shown that FRα

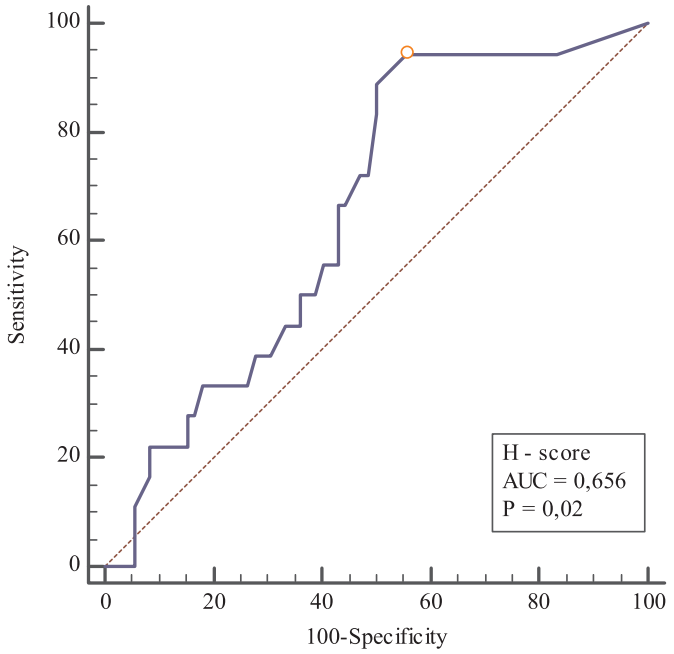

Fig. 2. ROC analysis of sensitivity, specificity and a cut-off value of H-score in determining the risk of disease recurrence.

is overexpressed in 80% of epithelial ovarian cancers and that it's expression significantly correlates with histologic grade and stage; in non-mucinous ovarian cancers, it shows a significant correlation with high tumor grade and contributes to worse survival [25]. In lung cancer, however, it is shown that the FRα expression is correlated with improved overall survival as well as 3-year disease-free survival. Also, studies have shown that the well-differentiated lung carcinomas have a higher expression of FRα, compared to low differentiated carcinomas [26,27]. The expression has also been studied in breast carcinoma and studies showed that higher expression correlates with the TNBC phenotype [18,19,28,29].

Intrigued by its higher expression in TNBC, we conducted this study on a cohort of 92 patients with primary TNBC, to evaluate its correlation with clinicopathological parameters and clinical survival, in order to distinguish patients who might have the advantage of potential FRα targeted therapies.

To evaluate the expression of FRα we used an H-score, that captures both the intensity of staining and the proportion of stained cells, and, for the purpose of this study, deemed the result of < 10 as negative expression. In the present study, 85% of patients expressed FRα, which is consistent with most studies that showed that TNBC has a higher incidence of FRα expression compared to ER/PR positive breast carcinomas [18,28,30]. This increased expression in TNBC is explained by the data from previous studies showing that ER negatively regulates the expression of FRα [19].

Several authors conducted research on the association of FRα expression and clinicopathologic parameters in breast carcinomas, and results are inconsistent. Aboulhagag et al. [31] showed in their research that FRα expression is significantly correlated with higher histologic grade (grade III), greater tumor size, and presence of lymphovascular

Table 3
ROC curve parameter considering the risk of disease recurrence.

Parameter	AUC	95% CI	Sensitivity	Specificity	Cut off value	Youden index	P
H-score	0,656	0,549–0,753	94,4	44,4	> 75	0,39	0,02*

AUC, area under the ROC curve; CI, confidence interval.
* Statistically significant (P < 0.05).

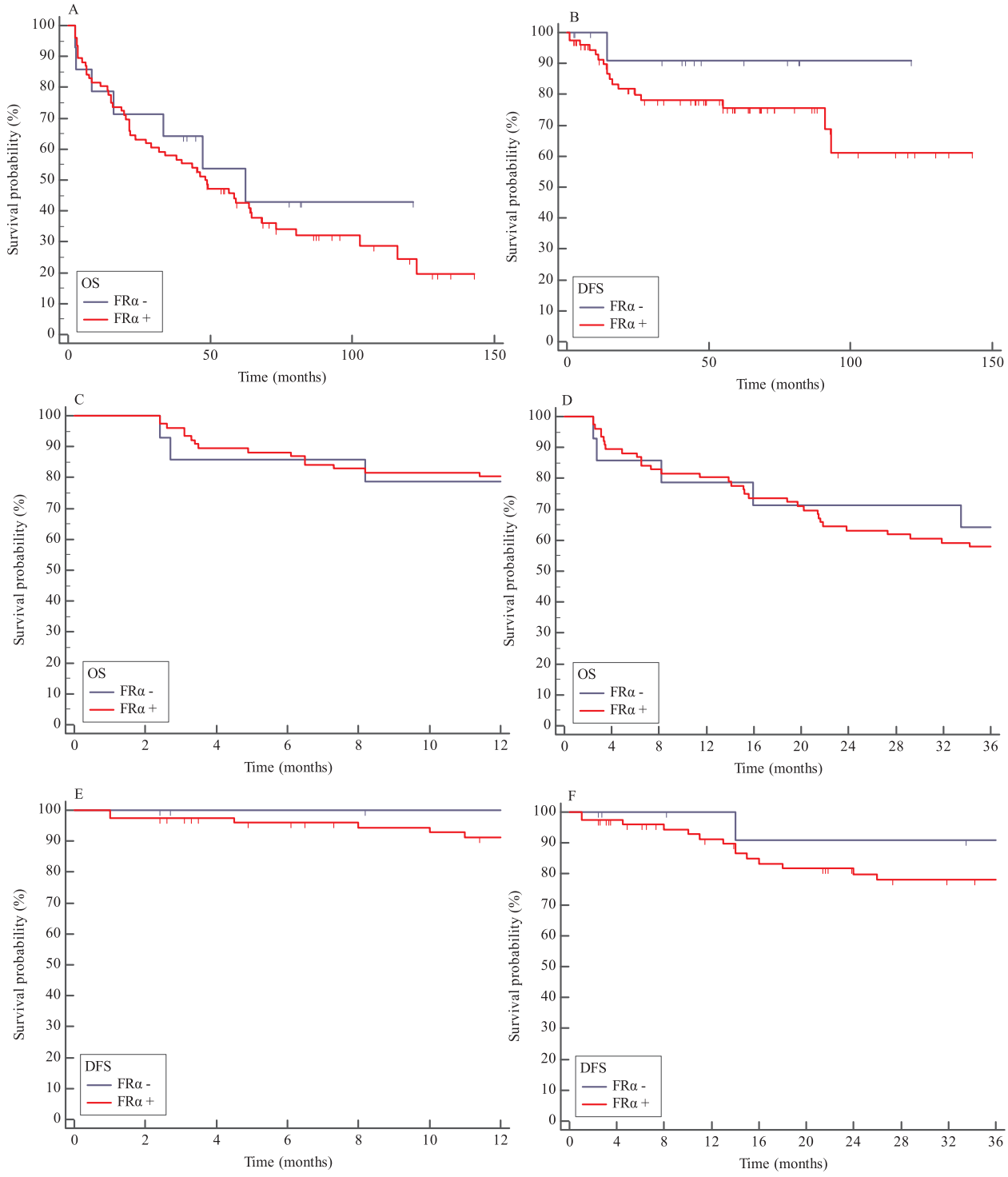

Fig. 3. Kaplan-Meier survival analysis OS (A) and DFS (B) according to FRα+/−; one-year (C) and three-year (D) survival according to FRα+/−; one-year (E) and three-year (F) DFS according to FRα+/−.

invasion. Similar results came from the study of Zhang et al. [30], which also showed a correlation with histologic grade, but found no correlation with tumor size. Our study also confirmed the correlation of FRα expression with histologic grade, but unlike previous studies, our study showed that grade III carcinomas have lower FRα expression, compared to grade II carcinomas. We believe that the discrepancies might arise in the sample size, as well as in the fact that the aforementioned authors correlated expression and clinicopathologic parameters within all breast cancer subtypes, while our study was based on TNBC subtype only. Further, this study found no significant association of FRα expression with lymph node involvement and lymphovascular invasion, which is consistent with the study from Necela et al. [19] that found no association with nodal status and age. In our study, we haven't found a significant correlation between the FRα and age of the patient, nor with the location of primary carcinoma (left/right breast).

In the research conducted by Ginter et al. [32], no significant association has been found between FRα expression and histologic type of tumor, in a cohort of primary TNBC. That is in contrast with our results,

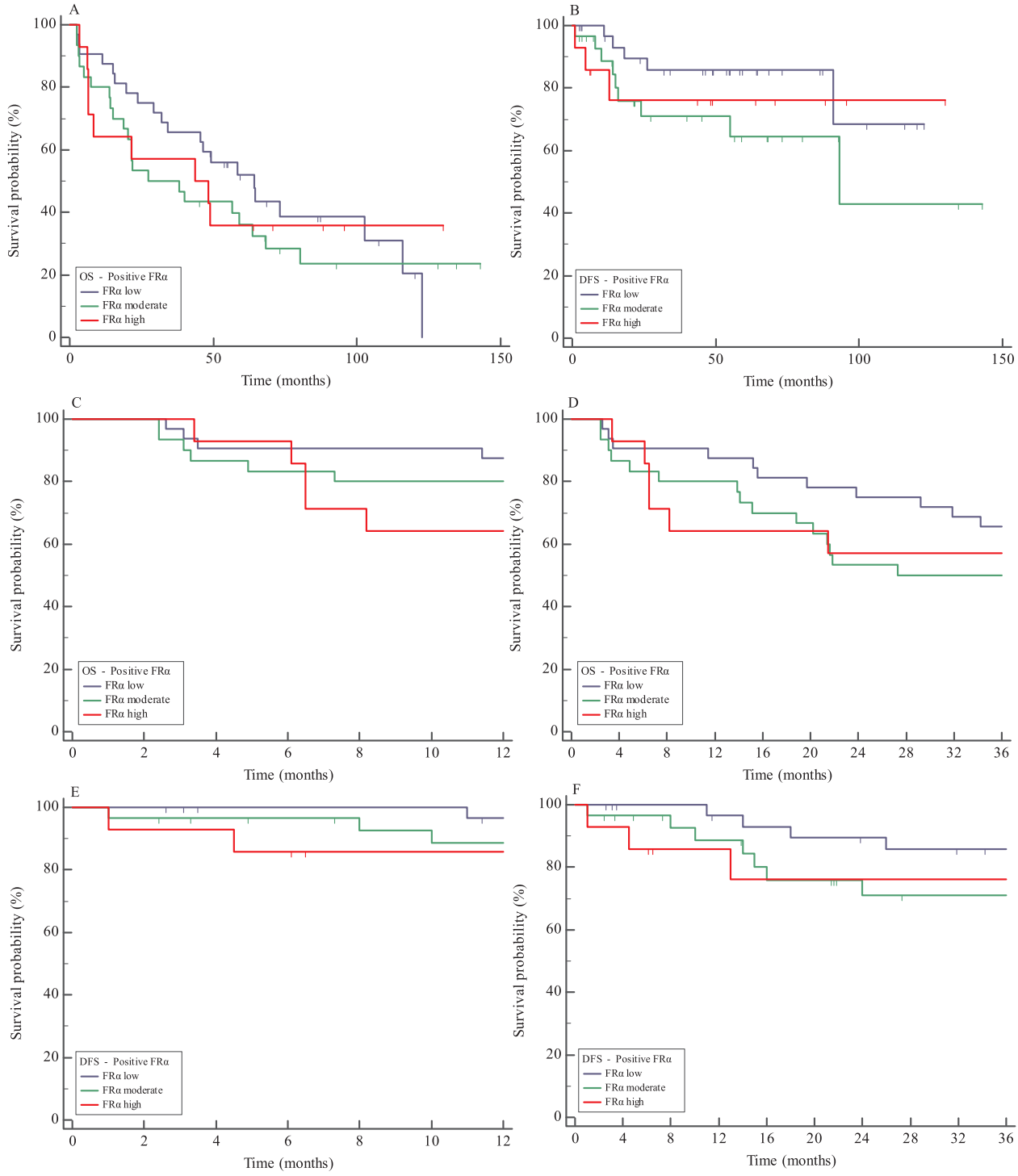

Fig. 4. Kaplan- Meier survival analysis OS (A) and DFS (B) according to positive values of FRα; one-year (C) and three-year (D) survival according to positive FRα subgroups; one-year (E) and three-year (F) DFS according to positive FRα subgroups.

which showed a significant association with lobular histology (P = 0,02). Although it must be considered that lobular histology was present in only two of our patients, this might indicate that TNBC patients with that histologic type, as well as those with grade II carcinoma, might benefit from potential FRα directed therapies, considering their higher FRα expression. Authors believe that a study involving a larger number of patients with this histologic type should be made to further support this claim.

Unfortunately, when exclusion factors were applied, mainly the evidence of metastatic disease prior to the surgical excision of the tumor and prior history of breast cancer, this study did not record any other histologic type of TNBC, except the invasive ductal and lobular carcinoma. Since significant association between FRα expression and lobular histology has been found, one can assume that involving other histologic types in the study could also produce significant results and authors agree that further study should be made, to test the correlation of FRα expression and histologic type of breast cancer.

By using Pearson's coefficient of correlation it was noticed that for higher values of Ki-67 H-score takes on lower values (r = −0,284 P = 0,006), suggesting that in the group of FRα-positive, TNBC, other

factors determine the malignant potential of the tumor and that it is not related to Ki-67 proliferative activity.

Interestingly, this study showed a significant correlation of FRα expression and disease relapse (whether in the form of locoregional relapse or distant metastasis). Using the Mann Whitney *U* test study found that values of H-score in patients that relapsed (with median of 127.5) were higher than in patients that had no relapse (median of 85) (P = 0.04). By using Cox univariate and multivariate analysis cutoff value of > 75 was found to be a statistically significant predictor for the risk of disease recurrence (OR = 1,005, P = 0,04). Given low specificity (44.4) cutoff of 75 would not necessarily be specific enough, although it is highly sensitive (94.4), in predicting which patients would relapse. These findings indicate that TNBC that express more FRα, could more likely develop the widespread disease and implies that in this subtype of breast cancer, FRα expression could be more related to metastatic tumor potential than with initial local spread (in axillary lymph nodes). Considering given data this study showed that lower grade TNBC, that are somehow more biologically aggressive, express more FRα, and as such pose a population that could benefit from potential FRα therapy.

Survival studies show conflicting results considering the association of FRα with the outcome. While one study found no association between FRα and OS in TNBC patients [33], other study from Norton and al. observed better survival in the majority of patients who expressed FRα [34] and two different studies found that FRα expression is significantly correlated with poorer DFS [30,32]. In our study, we found no statistically significant association of FRα expression with OS or DFS, and by using Kaplan-Meier curves we also found no association with one-year or three-year survival. Discrepancies in studies might arise from the different sample sizes as well as different scoring criteria for FRα positivity. We acknowledge that our study is limited by a relatively small sample size, and additional studies on a larger cohort of TNBC should be made to further address the issue of correlation of FRα expression and survival in TNBC.

5. Conclusion

The present study reports that FRα is highly expressed in triple negative breast cancer phenotype, in which its expression highly correlates with lobular histology as well as the histologic grade of the tumor. As such, by analyzing it immunohistochemically, we could pinpoint the patients that represent a significant subpopulation that could benefit from potential FRα targeted therapy. Importantly, the study showed that FRα H-score might be a significant predictor for the risk of disease recurrence, hence immunohistochemical identification of that score might help us identify the patients who are at risk of developing the widespread disease. Authors acknowledge that there are some limitations to this study. It is a retrospective study with not so high a number of patients which resulted in only 2 histologic types of breast cancer being analyzed, with a small number of invasive lobular carcinoma. The authors believe that validation in a larger cohort is needed to extend the present findings.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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