Triple negative breast cancer (TNBC) comprises 15–20% of all invasive breast cancer and is associated with a poor prognosis. As therapy options are limited for this subtype, there is a significant need to identify new targeted approaches for TNBC patient management. The expression of the folate receptor alpha (FRα) is significantly increased in patients with TNBC and is therefore a potential biomarker and therapeutic target. We optimized and validated a FRα immunohistochemistry method, specific to TNBC, to measure FRα expression in a centrally confirmed cohort of 384 patients with TNBC in order to determine if expression of the protein is associated with invasive disease-free survival (IDFS) and overall survival (OS). The FRα IHC demonstrated exceptional performance characteristics with low intra- and interassay variability as well as minimal lot-to-lot variation. FRα expression, which varied widely from sample to sample, was detected in 274 (71%) of the TNBC lesions. In a multivariable model adjusted for baseline characteristics, FRα expression was associated with improved IDFS (HR = 0.63, p = 0.01) but not with OS. The results demonstrate the potential of targeting the FRα in the majority of TNBC patients and suggest that variable expression may point to a need to stratify on FRα expression in clinical studies.

**Results**

The FRα-specific staining protocol demonstrated outstanding performance.

The antibody was tested at 1:50, 1:100, 1:200 dilutions on an optimization TMA. No staining was observed (i.e., H-scores were all 0) for all liver and spleen tissue (Supplementary Fig. S1). At an antibody concentration of 1:100, of the normal breast tissue cores (n = 18), H-scores ranged from 0 to 220, mean H-score = 20 ± 56 (±SD). At an antibody concentration of 1:100, of the serous ovarian tissue cores used as positive controls (n = 18), the H-score ranged from 190 to 300, mean H-score 245 ± 57. At 1:100, the H-scores for the 19 TNBC specimens ranged from 0 to 280. At this dilution, 3 of 19 (16%) tumors showed zero staining (H-score of 0 for all three replicate punches). Sixteen of 19 (84%) of patients had a mean H-score > zero (Supplementary Fig. S2).

**Discussion**

In the current study we developed and optimized an immunohistochemistry method to score FRα and used it in a centrally confirmed cohort of TNBC to test the association of FRα protein expression with invasive disease-free survival (IDFS) and overall survival (OS).
To characterize intra-assay variability, three adjacent 5-μm sections were taken from the blocks of 11 TNBC, three serous ovarian cancer, three liver and three spleen specimens to represent a range of intensities (based on observed H-scores from the optimization TMA). Three 5-μm sections were processed at antibody dilution of 1:100 in the same batch with the same reagents at the same time (Supplementary Fig. S3). Intra-assay variability across each of three 5-μm sections from the same specimen was 0, demonstrating high precision.

One 5-μm section, adjacent to those used in Supplementary Fig. S3, was taken from the blocks and stained on a separate day to those taken for intra-assay variability. Sections were processed at antibody dilution of 1:100 in the same batch with the same reagents, same technician and scored by the same pathologist. Linear regression analysis of H-scores on same samples (same core) processed at two different times showed a correlation of $R^2 = 0.86$ (Supplementary Fig. S4). Thus, the protocol demonstrated excellent intra-assay variation.

We observed high reproducibility between two different antibody lots 13J4007 and 13J4008, when testing the same 1:100 dilution lot of 13J4007 (used for all 384 TNBC patients in this study) against a range of dilutions of a newer lot, 13J4008, with correlations ranging $R^2 = 0.80–0.95$ (Supplementary Fig. S5).

To assess for FRα antigen stability, two adjacent 5-μm sections were taken from the blocks of TNBC patients: TN1, TN2, TN3, TN5, TN7, TN8, TN10, TN13, TN14, TN 17, and TN19, serous ovarian patients Sec1, Sec2, and Sec6, liver and spleen to represent a range of intensities (based on observed H-scores) at three different time points, day 0, day 15, and day 30. One section from each sample at each time point was stored at room temperature and a paired sample was stored at 4 °C. All sections were stained on day 30 with antibody from lot #13J4007 (used for all 384 TNBC patients in this study). We observed strong correlations between H-scores for the same core that was typed by the same pathologist 2 weeks apart with $R^2$ values of 0.93 and 0.88, respectively (Supplementary Fig. S6 panels A–C), highly similar to correlations observed for inter assay and antibody lot experiments described above. We also observed good correlation for samples stored at 4 °C ($R^2$ ranged 0.83–0.89) (Supplementary Fig. S6 panels D, E). It was concluded that FRα is stable for at least 30 days at ambient temperature and when refrigerated.

**Patient characteristics**

Once optimized and validated, we evaluated FRα expression in our study cohort which consisted of 384 women with centrally confirmed TNBC derived from our internal TNBC patient registry and repository. The patient characteristics are shown in Table 1. The mean age of participants was 56 years, 50% of patients had tumor size ≤2.0 cm, and 64% were node-negative. The median follow-up for these patients was 12.7 years (interquartile range: 8.9–18.5 years).

FRα is expressed in a high percentage of TNBCs

We observed strong correlations between H-scores for the same core that was typed by the same pathologist 2 weeks apart with $R^2$ values of 0.88, 0.94, 0.91, 0.87, and 0.85 for TMAs 1–5, respectively. For patients represented by multiple cores ($N = 370$), the correlation of H-scores was $R^2 = 0.68$. The mean H-score was 77 with 110 of 384 (29%) participants having H-Score value of 0 (Table 1). Representative images are shown in Fig. 1. The mean stromal TIL count was 27.6%. There was no statistically significant association between H-score and stromal TIL count ($p = 0.25$).

**Table 1.** Patient and tumor characteristics ($N = 384$).

| Parameter                          | Values       |
|-----------------------------------|--------------|
| Age (continuous)                  | Mean (SD) 55.6 (13.7) |
|                                    | Q1, median, Q3 45, 54.4, 65.8 |
|                                    | Range 29.3–88.4 |
| Menopausal status                 | Post 227 (59%) |
|                                   | Pre/Peri 157 (41%) |
| Tumor size                        | T1 (0.1–2.0 cm) 191 (49.7%) |
|                                   | T2 (2.1–5.0 cm) 169 (44%) |
|                                   | T3/4 (5.1–cm) 23 (5.99%) |
|                                   | Unknown 1 (0.26%) |
| Nodal status                      | N0 245 (64%) |
|                                   | N1 81 (21%) |
|                                   | N2 32 (8%) |
|                                   | N3 22 (6%) |
|                                   | NX 4 (1%) |
| Histology                         | Invasive carcinoma NST 253 (66%) |
|                                   | Metaplastic carcinoma NST 30 (8%) |
|                                   | Carcinoma with apocrine differentiation 27 (7%) |
|                                   | Carcinoma with medullary features 74 (19%) |
| Nottingham grade                  | 1 2 (0.521%) |
|                                   | 2 28 (7.29%) |
|                                   | 3 354 (92.2%) |
| KI-67 grouping                    | ≤15% 75 (19.5%) |
|                                   | 15.1–30% 62 (16.1%) |
|                                   | >30% 246 (64.1%) |
|                                   | Unknown 1 (0.26%) |
| Stromal TILs                      | 0–10% 125 (33%) |
|                                   | 10–20% 85 (22%) |
|                                   | 20–40% 86 (22%) |
|                                   | >40% 85 (22%) |
|                                   | Unknown 3 (1%) |
| FRα H-score (continuous)          | Mean (SD) 77.1 (85.5) |
|                                   | Q1, median, Q3 0, 49, 133 |
|                                   | Range 0–300 |
| FRα H-score group (zeros and rest by thirds) | 0 110 (28.6%) |
|                                   | 0.25–52.5 89 (23.2%) |
|                                   | 52.5–140 93 (24.2%) |
|                                   | 140–300 92 (24%) |
| Type of breast surgery            | Mastectomy 192 (50%) |
|                                   | Lumpectomy 192 (50%) |
| Adjuvant chemotherapy             | Yes 217 (56.5%) |
|                                   | No 118 (30.7%) |
|                                   | Unknown 49 (12.8%) |
| Adjuvant radiotherapy             | Yes 182 (47.4%) |
|                                   | No 146 (38%) |
|                                   | Unknown 56 (14.6%) |

**NST** no special type, **FRα** folate receptor alpha.
Increased FRα expression is associated with improved invasive disease-free survival (IDFS)

In a univariable analysis (Table 2), stromal TILs (HR = 0.88, per 10% increase, \( p = 0.002 \)), nodal status (overall \( p \)-value across all levels = 0.0006), and FRα H-scores (>0 vs 0; HR = 0.68; \( p = 0.02 \), Fig. 2) were associated with IDFS. The median IDFS (95% CI) for women with 0–10%, 10–20%, 20–40%, and >40% stromal TIL were 9.2 years (5.3–17.5), 10.9 years (3.8–NE (not evaluable)) 12.0 years (8.6–NE), and 20.4 years (11.8–NE), respectively.

In separate analyses of FRα H-scores, we compared the outcome of patients in each of the positive H-score tertiles (0.25–52.5, 52.5–140, and 140–300) against patients with zero expression (H-score 0) (Table 2). In patients with relatively low levels of FRα expression (H-scores 0.25–52.5 and 52.5–140), IDFS was significantly improved compared with patients with H-score of 0 (HR 0.55, \( p = 0.01 \) and HR 0.64, \( p = 0.05 \), respectively). The group of patients with the highest FRα expression (H-scores 140–300) did not show significantly improved survival relative to patients with no FRα expression, although the HR was in the same direction as the low FRα groups (HR 0.84, \( p = 0.4 \)).

In the multivariable analysis (Table 3), FRα H-score (>0 vs 0, \( p = 0.01 \)), adjuvant chemotherapy (vs no chemotherapy, \( p = 0.03 \)), stromal TILs (per 10% increment, \( p = 0.003 \)), and nodal status (\( p < 0.0001 \)) were significantly associated with IDFS.

FRα expression and overall survival (OS)

Univariable analyses of OS are shown in Table 2. Age (>65.8 compared with age < 45, \( p < 0.001 \)), nodal status (any nodal positivity compared with N0, \( p < 0.001 \)), adjuvant chemotherapy (compared with no chemotherapy, \( p = 0.04 \)), tumor size > 5.0 cm (compared with 0.1–2.0 cm, \( p = 0.02 \)), menopausal status (\( p < 0.001 \)), and stromal TILs (\( p = 0.001 \)) were associated with OS; with older age, post-menopausal, node-positive, low stromal TILs, and absence of adjuvant chemotherapy all being associated with decreased OS.

FRα H-score was not associated with OS (overall \( p \)-value = 0.11 comparing the four groups of H-scores). The patient group with H-score values 0.25–52.5 appeared to have longer survival times when compared with participants with values of 0. However, this association was not seen in patients with higher H-scores. Comparing H-scores of >0 vs 0 was also not statistically significant (HR = 0.75, \( p = 0.10 \)) but showed a similar trend to that observed with IDFS (Fig. 3).

In the multivariable analysis (Table 3), FRα H-scores of >0 vs 0 were not statistically associated with OS (HR = 0.87, \( p = 0.46 \)). Age (across all levels compared with age < 45, \( p < 0.04 \)), nodal status (across all levels compared with N0, \( p < 0.001 \)), adjuvant chemotherapy (all levels compared with no chemotherapy, \( p = 0.002 \)),

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**Fig. 1** Representative FRα staining of triple negative breast tumors (20×). Membrane staining was scored as negative (0), weak (1+), moderate (2+), and strong (3+). The percent of cells within each tissue core stained at each intensity were recorded to calculate an H-score for each sample. The H-score for staining each sample was defined as: H-score = 0* (% at 0) + 1* (% at 1+) + 2* (% at 2+) + 3* (% at 3+).

- **A** H-score = 0: (3 × 0 + 2 × 0 + 3 × 0).
- **B** H-score = 50: (3 × 0 + 2 × 0 + 1 × 50).
- **C** H-score = 120: (3 × 0 + 2 × 20 + 1 × 80).
- **D** H-score = 140: (3 × 0 + 2 × 40 + 1 × 60).
- **E** H-score = 200: (3 × 20 + 2 × 60 + 1 × 20).
- **F** H-score = 300: (3 × 100 + 2 × 0 + 1 × 0).

Scale bar is 50 µm.
Table 2. Univariable analysis of patient characteristics and FRα H-score with invasive disease-free survival and overall survival.

|                      | IDFS HR (95% CI) | P-value | OS HR (95% CI) | P-value |
|----------------------|------------------|---------|---------------|---------|
| **Age**              |                  |         |               |         |
| <45.0                | 1.0 (ref)        |         | 1.0 (ref)     |         |
| 45.0–54.3            | 0.85 (0.54–1.32) | 0.46    | 0.83 (0.48–1.41) | 0.483  |
| 54.4–65.7            | 0.92 (0.58–1.46) | 0.72    | 1.52 (0.93–2.49) | 0.091  |
| 65.8–88.4            | 1.44 (0.92–2.26) | 0.11    | 3.01 (1.92–4.70) | <0.001 |
| **Menopausal status**|                  |         |               |         |
| Pre/peri             | 1.0 (ref)        |         | 1.0 (ref)     |         |
| Post                 | 1.31 (0.94–1.81) | 0.11    | 2.37 (1.66–3.38) | <0.001 |
| **Tumor size**       |                  |         |               |         |
| T1 (0.1–2.0 cm)      | 1.0 (ref)        |         | 1.0 (ref)     |         |
| T2 (2.1–5.0 cm)      | 0.94 (0.68–1.32) | 0.73    | 1.11 (0.80–1.55) | 0.53   |
| T3/4 (5.1+ cm)       | 1.7 (0.94–3.06)  | 0.08    | 1.95 (1.10–3.46) | 0.02   |
| **Nodal status**     |                  |         |               |         |
| N0                   | 1.0 (ref)        |         | 1.0 (ref)     |         |
| N1                   | 1.36 (0.92–2.02) | 0.13    | 1.59 (1.06–2.40) | 0.03   |
| N2                   | 1.84 (1.1–3.06)  | 0.02    | 2.64 (1.63–4.28) | <0.0001 |
| N3                   | 3.44 (2.6–2.7)   | <0.0001 | 3.59 (2.12–6.09) | <0.0001|
| NX                   | 3.58 (0.88–14.60)| 0.08    | 6.96 (2.53–19.20)| 0.0002 |
| **Histology**        |                  |         |               |         |
| Invasive carcinoma NST | 1.0 (ref)    |         | 1.0 (ref)     |         |
| Metaplastic carcinoma NST | 0.96 (0.53–1.74) | 0.88  | 0.63 (0.30–1.28) | 0.20  |
| Ca. with apocrine differentiation | 1.15 (0.64–2.05) | 0.64 | 1.11 (0.64–1.94) | 0.71  |
| Ca. with medullary features | 0.60 (0.38–0.96) | 0.03 | 0.60 (0.38–1.28) | 0.03  |
| **Nottingham grade** |                  |         |               |         |
| 1                    | 1.0 (ref)        |         | 1.0 (ref)     |         |
| 2                    | 0.49 (0.11–2.14) | 0.34    | 0.568 (0.13–2.49) | 0.45  |
| 3                    | 0.32 (0.08–1.32) | 0.12    | 0.398 (0.10–1.61) | 0.2   |
| **Ki-67 grouping**  |                  |         |               |         |
| ≤15%                 | 1.0 (ref)        |         | 1.0 (ref)     |         |
| 15.1–30%             | 0.76 (0.44–1.33) | 0.34    | 0.56 (0.32–1.00) | 0.05  |
| >30%                 | 1.06 (0.71–1.60) | 0.77    | 0.84 (0.57–1.24) | 0.38  |
| **Stromal TILs (per 10% increment)** | 0.88 (0.81–0.95) | 0.002 | 0.88 (0.81–0.95) | 0.001 |
| **FRα H-score**      |                  |         |               |         |
| 0                    | 1.0 (ref)        |         | 1.0 (ref)     |         |
| 0.25–52.5            | 0.55 (0.35–0.88) | 0.01    | 0.59 (0.37–0.93) | 0.02  |
| 52.5–140             | 0.64 (0.41–1.00) | 0.05    | 0.79 (0.51–1.21) | 0.28  |
| 140–300              | 0.84 (0.56–1.26) | 0.4     | 0.901 (0.60–1.38) | 0.65  |
| **FRα H-score ≤49.375 (median)** | 1.0 (ref) |         | 1.0 (ref)     |         |
| >49.375 (median)     | 0.94 (0.69–1.30) | 0.73    | 1.07 (0.78–1.47) | 0.67  |
| **Surgery type**     |                  |         |               |         |
| Lumpectomy           | 1.0 (ref)        |         | 1.0 (ref)     |         |
| Mastectomy           | 1.06 (0.77–1.46) | 0.72    | 1.35 (0.97–1.88) | 0.07  |
| **Adjuvant chemotherapy** |             |         |               |         |
| No                   | 1.0 (ref)        |         | 1.0 (ref)     |         |
| Yes                  | 0.79 (0.56–1.10) | 0.16    | 0.7 (0.49–0.99) | 0.04  |
| Unknown              | 1.18 (0.51–2.74) | 0.71    | 1.57 (0.96–2.59) | 0.07  |
| **Adjuvant radiotherapy** |           |         |               |         |
| No                   | 1.0 (ref)        |         | 1.0 (ref)     |         |
| Yes                  | 0.94 (0.67–1.30) | 0.7     | 0.73 (0.52–1.04) | 0.08  |
| Unknown              | 1.06 (0.42–2.64) | 0.91    | 1.5 (0.92–2.45) | 0.1   |

NST no special type, FRα folate receptor alpha.
studies evaluated the association of FRα expression in patients with breast cancer. To date, three published studies that found an association between FRα expression and worse DFS in 76 TNBC patients (HR, 2.61, \( p = 0.0497 \)); however, in that study 80.3% of the TNBC cases evaluated were negative for FRα (defined as ≥5% positive staining). \(^{12} \) Although each of these studies used different criteria for scoring FRα positivity, between 20 and 30% of the patient samples were scored as FRα expressing and these patients demonstrated worse survival. This is in contrast with our study in which we observed better survival in ~71% of patients who expressed FRα at some level and we did not observe a worse outcome in the 30% of patients with the highest levels of FRα.

To put into context these conflicting findings, we draw a parallel to studies of FRα expression and survival in ovarian carcinoma which also demonstrate the importance of sample size, histological subtype, central review of IHC and further evidence that FRα positivity is associated with improved survival. A relatively small study of ovarian carcinoma (\( N = 91 \)) showed association of increased FRα gene expression with worse survival,\(^ {16} \) two IHC studies of mixed histological subtypes (\( N = 186 \) and \( N = 361 \), respectively) showed no association of FRα expression with survival\(^ {17,18} \) and a subsequent larger study from the Ovarian Tumor Tissue Analysis consortium, demonstrated association of FRα positivity with improved survival, specifically in patients with high grade serous ovarian carcinoma (\( N = 1422 \)).\(^ {19} \) The Kobel study,\(^ {19} \) which showed improved survival in patients who had FRα expression was the largest study of FRα expression in ovarian cancer, the IHC was centrally reviewed and they used a similar scoring of FRα expression to our own, in that patients with absent or weak (<1%) staining were defined as negative and all other patients were recorded as positive. Similar criteria of FRα positivity were also used in a study of non-small cell lung cancer, in which H-scores ≥ 20 were associated with prolonged PFS (5.5 vs. 3.4 months; HR = 0.61; \( p = 0.0254 \)) and improved OS (12.1 vs. 6.4 months; HR = 0.57; \( p = 0.0076 \)).\(^ {20} \) Finally, FRα positive expression is associated with better prognosis in a second study of non-small cell lung cancer\(^ {21} \) and in pancreatic ductal adenocarcinoma.\(^ {22} \)

There are two possible mechanistic links between expression of FRα and better prognosis. First, one could hypothesize that tumors with positive FRα expression may be more susceptible to receptor-targeted therapies. Second, FRα expression may be associated with a more favorable tumor microenvironment or immune phenotype.
expressing FRα are more sensitive to chemotherapy. Huang showed that SKOV3 ovarian cancer cells overexpressing FRα were significantly more sensitive to cisplatin than controls.23 In addition, one could hypothesize that chemotherapy releases FRα antigens driving an immune response. This is consistent with our prior data where we prospectively tested for immunity in both breast and ovarian cancer patients using a panel of FRα-derived peptides representing potential T-cell epitopes.24 In that study, more than 70% of patients demonstrated immunity to at least one FRα peptide.

The limitations of our study are the retrospective nature and that patients were treated heterogeneously (56% of patients were treated with chemotherapy and 31% did not receive chemotherapy). However, this heterogeneity did allow us to determine the

| Table 3. Multivariable analysis of FRα H-score with invasive disease-free and overall survival. |
|------------------------------------|-----------|-----------------|-----------------------------------|
|                                    | IDFS      | P-value         | OS                                | P-value |
| **Age**                            |           |                 |                                   |         |
| <45                                | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| 45–54.3                            | 0.65 (0.39–1.08) | 0.09            | 0.62 (0.33–1.17) | 0.14   |
| 54.4–65.7                          | 0.55 (0.27–1.10) | 0.09            | 0.80 (0.36–1.77) | 0.58   |
| 65.8–88.4                          | 0.73 (0.35–1.53) | 0.41            | 1.29 (0.58–2.88) | 0.53   |
| **Menopausal status**              |           |                 |                                   |         |
| Pre/Peri                           | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| Post                              | 1.66 (0.95–2.91) | 0.08            | 2.00 (1.02–3.91) | 0.04   |
| **Tumor size**                    |           |                 |                                   |         |
| T1 (0.1–2.0 cm)                    | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| T2 (2.1–5.0 cm)                    | 1.17 (0.81–1.71) | 0.4             | 1.35 (0.92–1.96) | 0.12   |
| T3/4 (5.1+ cm)                     | 1.84 (0.92–3.69) | 0.08            | 1.90 (0.97–3.74) | 0.06   |
| **Nodal status**                  |           |                 |                                   |         |
| N0                                 | 1.0 (ref) | 0.008           |                                   | 1.0 (ref) |
| N1                                 | 1.90 (1.18–3.04) | 0.008           | 2.72 (1.67–4.43) | <0.001 |
| N2                                 | 2.33 (1.27–4.29) | 0.007           | 3.54 (1.96–6.37) | <0.001 |
| N3                                 | 6.02 (3.05–11.90) | <0.001         | 8.95 (4.65–17.24) | <0.001 |
| NX                                 | 2.44 (0.53–11.36) | 0.25            | 3.77 (1.21–11.74) | 0.02   |
| **Histology**                      |           |                 |                                   |         |
| Invasive carcinoma NST             | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| Metaplastic carcinoma NST          | 0.82 (0.43–1.58) | 0.55           | 0.50 (0.23–1.09) | 0.08   |
| Ca. with apocrine differentiation  | 0.53 (0.22–1.28) | 0.16           | 0.38 (0.16–0.91) | 0.03   |
| Ca. with medullary features        | 0.83 (0.49–1.41) | 0.49           | 0.80 (0.47–1.37) | 0.42   |
| **Nottingham grade**               |           |                 |                                   |         |
| 1                                  | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| 2                                  | 1.10 (0.23–5.27) | 0.9             | 1.21 (0.25–5.91) | 0.81   |
| 3                                  | 0.54 (0.11–2.55) | 0.44           | 0.83 (0.18–3.84) | 0.81   |
| **Ki-67 grouping**                 |           |                 |                                   |         |
| ≤15%                               | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| 15.1–30%                           | 0.94 (0.51–1.72) | 0.83           | 0.78 (0.42–1.45) | 0.43   |
| >30%                               | 1.28 (0.77–2.11) | 0.34           | 0.93 (0.58–1.49) | 0.75   |
| **Stromal TILs (per 10% increment)** | 0.86 (0.78–0.95) | 0.003       | 0.86 (0.78–0.95) | 0.004 |
| **FRα H-score**                    |           |                 |                                   |         |
| 0                                  | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| >0                                 | 0.63 (0.44–0.91) | 0.01           | 0.87 (0.60–1.26) | 0.46   |
| **Adjuvant chemotherapy**          |           |                 |                                   |         |
| No                                 | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| Yes                                | 0.61 (0.39–0.95) | 0.03           | 0.44 (0.28–0.71) | <0.001 |
| Unknown                            | 1.27 (0.34–4.73) | 0.73           | 1.07 (0.34–3.33) | 0.91   |
| **Adjuvant radiotherapy**          |           |                 |                                   |         |
| No                                 | 1.0 (ref) |                 |                                   | 1.0 (ref) |
| Yes                                | 0.87 (0.51–1.48) | 0.62           | 0.85 (0.50–1.43) | 0.53   |
| Unknown                            | 1.12 (0.28–4.56) | 0.87           | 0.87 (0.28–2.68) | 0.81   |

NST no special type, FRα folate receptor alpha.
prognostic role of FRα in patients treated with surgery alone (no
adjuvant chemotherapy) and separately in patients who were
treated with chemotherapy, in which we observed that FRα H-
scores of >0 were associated with better IDFS in both of these
groups. Thus, although our study did not identify why FRα
expression is associated with better outcome, our observation that
FRα is also prognostic in untreated TNBC, generates a new
hypothesis, that endogenous immune responses against FRα may
also drive prognosis in TNBC. However, evaluation of a larger
randomized cohort would be needed to test this hypothesis and if
there is any interaction between chemotherapy and FRα expres-
sion in determining outcome, for which our optimized assay of
FRα expression would be a useful tool.

What may be the most important finding in this study is that
while >70% of TNBC patients showed at least some positivity for
FRα, patients with FRα positive TNBC still exhibited disease
recurrence, albeit at a lower rate compared with FRα negative
patients, suggesting a substantial need to improve the therapeutic
outcomes for this group. Regarding treatment strategies for this
subset of patients, there are now several therapies directed at FRα
which include monoclonal antibodies alone such as farletuzumab
or as drug conjugates such as MOv18-IgG1 (and anti-FRα antibody
conjugated with a Src inhibitor25), FRα engineered chimeric
antigen receptor (CAR) T cells26 and a vaccine-based approach.24
In the present cohort, IDFS in patients who were positive for FRα
was still declining after 10 years such that approaches that
generate a durable response may be more appropriate. CAR-
modeled T cells, for example, have the capacity to persist as
memory cells in vivo27,28 although recent data demonstrated that
FRα CAR T cells mediated antitumor activity against established
TNBC tumor when FRα is expressed at higher levels,26 which has
significant implications for pre-selection of TNBC patients based
on accurately defined FRα expression. Recent phase I clinical data
from our group demonstrated that a FRα peptide vaccine elicited
a durable (at least 12 months) T-cell response to the FRα peptides
in 90% of patients, including both breast and ovarian cancer.24
Therefore, further augmenting immune responses to patients with
TNBC may be of substantial therapeutic relevance, and a
randomized phase II trial (including correlative studies of FRα
expression with high performance IHC assay) is ongoing to test a
FRα vaccine in patients with high risk, resected TNBC
(NCT03012100).

METHODS
Ethics approval and consent to participate
All breast cancer specimens were collected according to a protocol that
was approved by the Mayo Clinic Institutional Review Board (IRB). The
study was conducted in accordance with the U.S. Common rule with
written, informed consent being obtained from each participating patient.

Tissue microarrays (TMAs)
A tissue microarray (TMA) was constructed for the purpose of antibody
optimization consisting of 1 mm core punches from deidentified waste
tissue: 8 from liver and 8 from spleen, intended as negative controls; 3
replicate punches of each of 19 TNBC breast tumors; 3 replicate punches of
each of 6 normal breast tissues and 3 replicate punches of each of 6 serous
ovarian cancer, tissues intended as positive controls. Samples from this
TMA and 5-μm sections from the same patients were used to optimize and
test antibody dilution, intra- and interassay variability, reproducibility
between different antibody lots and antigen stability.

Five TMAs with 1.0 mm cores, derived from TNBC surgical specimens,
were constructed by the Mayo Clinic Pathology Research Core. Two cores
from each tumor specimen were included per array and each array
included controls of liver, normal breast, tonsil, cervix, and placenta. Four
TMAs included 88 patient specimens and one TMA included 57 specimens.
The arrays were created using the semi-automated Alphelys (Plaisir,
France) Minicore tissue arrayer.

Immunohistochemistry
Immunohistochemistry (IHC) was performed on FFPE tissue microarrays
using a MACH4 Universal HRP-Polymer Detection Kit (Biocare Medical,
Pancheco, CA) as previously described.29 FFPE TMA specimens were
sectioned at 5 μm, placed on positively-charged glass slides and heated at
60°C for at least 1 h. Slides were deparaffinized in sequential baths of
xylene, transferred to sequential baths of 100% ethanol, followed by
sequential baths of 95% ethanol and then rinsed in deionized (DI) water.
The IHC procedure involves pretreatment of slides in Diva heat-induced epitope retrieval solution (Biocare Medical) inside a pressurized decloaking chamber with DI water and a pressurized incubation period at elevated temperature (125 °C at 16 psi for 30 s) followed by a 15 min of cooling to 95 °C. The slides were then cooled at room temperature, washed in sequential baths of Tris buffered Saline (0.1% Tween-20 wash buffer (TBST). Slides were blocked using Peroxidase-1 blocking solution (Biocare Medical) for 15 min, and then Universal Polymer-HRP reagent (Biocare Medical) for 20 min. After additional TBST washes, slides were incubated with a 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (Dako), rinsed and counter-stained with hematoxylin. Slides were washed with water, dehydrated with sequential baths each of 95 and 100% ethanol and then sequential baths of xylene before coverslips were applied.

IHC scoring
Digital images of the stained TMA slides were obtained using an Aperio ScanScope Image Scanner (Aperio Technologies, Vista, CA). TMA sections were evaluated using a semi-quantitative scoring method (Fig. 1). A pathologist (BY) scored membrane staining as negative (0), weak (1−), moderate (2−), and strong (3+) membrane staining. The percent of cells within each tissue core stained at each intensity were recorded to calculate an H-score for each sample. The H-score is a weighted score that captures both the proportion of positively stained cells and the intensity of staining, and thus is more representative of staining of the entire tumor section. The H-score for staining each sample was defined as: H-score = 0* (% at 0) + 1* (% at 1−) + 2* (% at 2−) + 3* (% at 3+). H-scores for duplicate patient cores were averaged. Duplicate patient cores were available for 370 patients with only 14 patients having a single core. For the 384 TNBC patients, each TMA was scored once by the study pathologist (BY) and then scored again with only 14 patients having a single core. The data supporting supplementary Figs 1–6 are available as part of the supplementary information.

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AUTHOR CONTRIBUTIONS

Conception and design: K.L.K., N.N., and B.M.N.; development of methodology: K.L.K., N.N., B.M.N., B.Y., X.G., A.N., and E.S.; acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L.K., N.N., M.P.G., J.N.J., F.J.C., E.A.P., H.L., K.J.R., M.C.L., J.M.C., J.C.B., R.A.L.F., K.R.K., and D.W.V.; analysis and Interpretation of data: K.L.K., N.N., B.M.N., D.W.H., and M.C.P.; writing, review, and/or revision of the paper: N.N., K.L.K., M.P.G., J.N.J., F.J.C., E.A.P., X.C., H.L., K.J.R., M.C.L., J.M.C., R.A.L.F., K.R.K., and D.W.V.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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