review articles

Meta-Analysis of Prevalence of Triple-Negative Breast Cancer and Its Clinical Features at Incidence in Indian Patients With Breast Cancer

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abstract

PURPOSE Breast cancer is the most common cancer in women in India, with higher incidence rates of aggressive subtypes, such as triple-negative breast cancer (TNBC).

METHODS A systematic review was performed to compute pooled prevalence rates of TNBC among patients with breast cancer, and clinical features at presentation were systematically compared with non-TNBC in an Indian cohort of 20,000 patients.

RESULTS Combined prevalence of TNBC among patients with breast cancer was found to be on the higher side (27%; 95% CI, 24% to 31%). We found that the estrogen receptor (ER) expression cutoff used to determine ER positivity had an influence on the pooled prevalence and ranged from 30% (ER/progesterone receptor [PR] cut ff at 1%) to 24% (ER/PR cutoff at 10%). Odds for TNBC to present in the younger age-group were significantly higher (pooled odds ratio [OR], 1.35; 95% CI, 1.08 to 1.69), with a significantly younger mean age of incidence (weighted mean difference, –2.75; 95% CI, –3.59 to –1.92). TNBC showed a significantly higher odds of presenting with high grade (pooled OR, 2.57; 95% CI, 2.12 to 3.12) and lymph node positivity (pooled OR, 1.39; 95% CI, 1.21 to 1.60) than non-TNBC.

CONCLUSION Systematic review and meta-analysis of 34 studies revealed a high degree of heterogeneity in prevalence of TNBC within Indian patients with breast cancer, yet pooled prevalence of TNBC is high in India. High proportions of patients with TNBC present with aggressive features, such as high grade and lymph node positivity, compared with patients without TNBC. We emphasize the need for standardized methods for accurate diagnosis in countries like India.

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INTRODUCTION

Breast cancer is the most common cancer in India, with the highest numbers of new cancer incidence per year (14%) and with a high incidence-to-mortality ratio (approximately 50%) according to GLOBOCAN 2018.[1] At present, breast cancer is classified into 4 molecular subtypes on the basis of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Positive expression of ER/PR and/or HER2 determines the ER-positive and/or HER2-positive subtype, while absence of ER, PR, and HER2 expression defines triple-negative breast cancer (TNBC).[2] Both, ER-positive and HER2-positive subtypes are effectively and routinely treated with respective targeted therapy.[3] In contrast, TNBCs lack targeted therapy and are still treated with systemic chemotherapy drugs. In addition, TNBCs tend to present with more aggressive clinical features[4] and tend to recur earlier and with higher frequency, which make them a most aggressive subtype of breast cancer.[5,6]

TNBC incidence in the West is at 12.2%-13% of all breast cancers,[4,6] with the highest prevalence in Blacks (22.5%-23.7%).[4,6] In India, several reports have suggested that TNBC incidence is higher and up to 31%.[7,8]

Having a higher incidence of TNBC may translate into a higher proportion of the aggressive disease that is clinically difficult to target, which contributes to higher mortality rates in India. Moreover, there is a high degree of variability in TNBC prevalence among individual studies.[7,8] We conducted a systematic review and meta-analysis to assess the effect of detection method for ER/PR positivity that determines triple-negative status of the disease because such methods are reportedly varied across centers in India.[9,10] Clinical features of TNBC and non-TNBC at incidence, such as age, grade, and lymph node involvement, were systematically compared with the understanding of whether TNBC in Indian cohorts present with a higher degree of aggressive features, as has been observed in the West.[6]

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

The meta-analysis systematically compared prevalence of triple-negative breast cancer (TNBC) in a large cohort of 20,000 Indian/Indian-origin patients from 34 studies.

Knowledge Generated

Indian patients with TNBC present with high rates (27%) of prevalence, although with a high degree of variability. To our knowledge, this is the first time a possible source of variability in TNBC prevalence among the studies has been objectively analyzed. Our study reveals and emphasizes the need for standardized methods for a standardized diagnostic protocol across the country.

Relevance

Even with the variable prevalence, patients with TNBC in India present at a significantly younger age compared with patients without TNBC and with a higher odds ratio of high-grade disease and lymph node involvement. Understanding the high rates of prevalence and clinical features of the most aggressive, triple-negative subtype may help to clarify and better interpret breast cancer outcomes in India.

METHODS

Search Criteria

The key terms used to search for the breast cancer reports in Indian cohorts were as follows: breast cancer, breast carcinoma, triple negative, ER, PR, HER2, TNBC, and Indian or India. The studies that were peer reviewed and listed in PubMed until October 2019 were included. To be certain that breast cancer studies with patients from India or of Indian origin were included in the analysis; individual studies/reports were manually curated for the following: studies conducted at and published from an Indian center (assuming that all the patients were of Indian origin) or studies conducted in countries other than India, with data clearly annotated for Indian-origin patients. With these inclusion criteria, 49 studies were identified[7-9,11-55] (Data Supplement).

Exclusion Criteria

Of the 49 studies identified, those that did not mention criteria for defining HER2 positivity or negativity, subtype prevalence, or cohort details; had information missing for part of the cohort; or were review articles were excluded. In total, 15 articles were excluded for reasons shown in Figure 1. The remaining 34 studies were considered for the systematic comparison between TNBC and non-TNBC for prevalence and clinical features at incidence, such as age, grade, and lymph node involvement.

Quality Assessment

Quality of the studies was assessed independently by 2 authors while following Newcastle-Ottawa Scale criteria for selection, comparability, and outcome. Consensus scores for each study are listed in the Data Supplement. Overall, 33 studies scored good for quality, with 3 stars in the selection domain, 2 stars in the comparability domain, and 2 stars in the outcome/exposure domain. One study scored fair for quality, with 2 stars in the selection domain.[36]

Data Extraction

For the articles included in the study, data were independently extracted by 2 investigators (A.K. and N.P.) for cohort number, ER/PR and HER2 expression reported by immunohistochemistry (IHC) while following standard guidelines given by ASCO-College of American Pathologists (CAP); TNBC and non-TNBC; number of patients; age at incidence; grade; and lymph node involvement. Extracted data were confirmed for each study (M.K).

In total, 34 studies reported the cohort numbers for patients with breast cancer with the molecular subtypes (Data Supplement). These studies were screened for their assessment of ER/PR status. According to 2010 ASCO-CAP guidelines, > 1% ER/PR expression is considered positive.[56] Before these guidelines, > 5%-10% ER/PR expression was set as a cutoff for ER/PR positivity.[57] On the basis of the reports, we observed that it has taken time to implement the guidelines across India, and a few articles published later than 2010 still followed the older guidelines. To assess the impact of this variability in defining TNBC status, we evaluated each study with respect to the ER expression cutoff used. First, all the studies that followed ASCO-CAP guidelines were evaluated here. The studies were segregated into 3 subgroups as follows: the ones that used 1% expression as cutoff to define ER/PR positivity (subgroup 1; n = 15), the ones that used 5%-10% expression as cutoff (subgroup 2; n = 5), and the ones that did not mention the percent expression cutoff or did not refer to the year of ASCO-CAP guidelines but did refer to ASCO-CAP guidelines (subgroup 3; n = 14).

Method for HER2 positivity assessment was also screened. All the studies evaluated here referred to the ASCO-CAP guidelines to determine HER2 positivity. The studies in subgroups 1 and 2 either referred to ASCO-CAP 2007 guidelines (n = 5) or determined HER2 positivity with HER2 IHC scores of 3+ or 2+ with positive fluorescence in situ

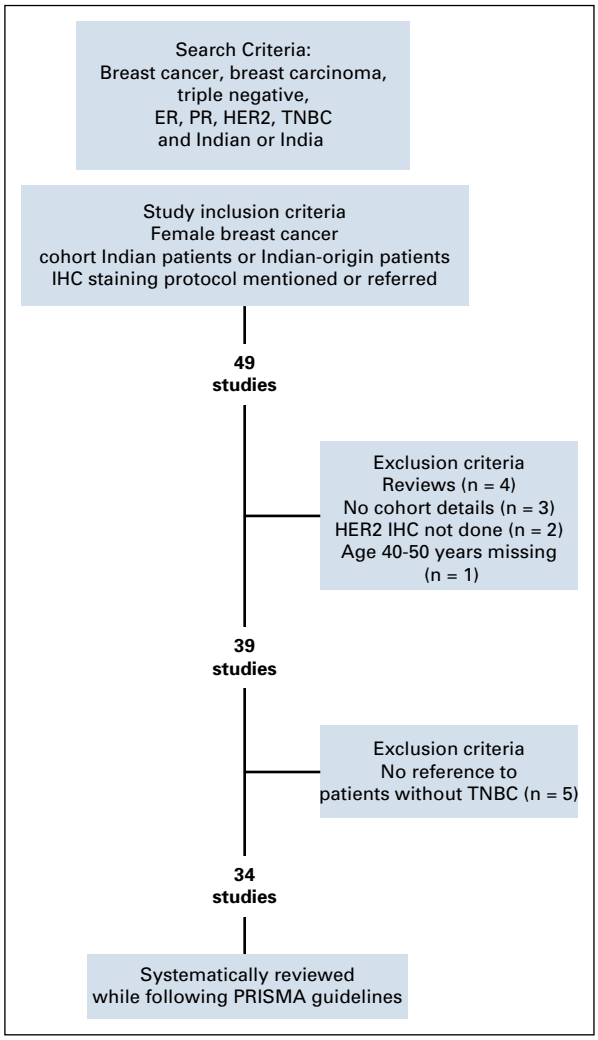


FIG 1. Search and inclusion/exclusion criteria of the studies. The flowchart depicts search criteria used to select the studies with breast cancer cohorts of Indian and/or Indian-origin patients. Exclusion and inclusion criteria are explained and led to the inclusion of 34 studies in the systematic review. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2, IHC, immunohistochemistry; PR, progesterone receptor; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; TNBC, triple-negative breast cancer.

hybridization (FISH; n = 14). The studies from subgroup 3 referred to ASCO-CAP guidelines without referring to the year, except for 3 studies that defined HER2 positivity with HER2 IHC scores of 3+ or 2+ with positive FISH.

Twenty-three of 39 studies reported age at incidence either as mean or as grouped by younger and older age. Mean age at incidence was reported by 12 studies, and 8 studies reported age-groups for patients with or without TNBC. Fourteen of 34 studies reported grade and lymph node status for patients with and without TNBC. In these 14 studies, grade was grouped into low (grade 1 and 2) and high (grade 3) categories. Lymph node involvement was referred to as positive when ≥ 1 lymph nodes were reported to be involved on the basis of a pathologic report, as mentioned in the studies.

Statistical Analysis

The reported number of patients with TNBC within the breast cancer total cohort of a given study was used to calculate TNBC proportions. These proportions were logit transformed to calculate effect size and study weight. Individual effect sizes and study weights were then pooled in fixed- and random-effects models and back transformed to proportions. The analysis was done on the basis of the inverse variance method, using the DerSimonian-Laird estimator for τ^2 and Clopper-Pearson CI as described in Wang et al.[58] Data were analyzed using the metafor (2.1.0) package[59] in R version 3.6.1[60] for Windows (Microsoft Corporation, Redmond, WA).

Moderator analysis allowed us to test for sources of heterogeneity in the pooled analysis. We used the reported IHC cutoff for ER positivity as the moderator for subgroup analysis. The 3 subgroups created for ER positivity were those described in the Data Extraction section. These subgroups were analyzed individually using a random-effects model, which assumed a difference between study variance (τ^2) across subgroups. Finally, the subgroups were pooled using a mixed-effects model, and the moderator effect was assessed by Wald test.

The statistical significance for the difference in mean age at incidence for 12 studies was analyzed using *t* test. Of those 12 studies, 9 reported a mean age with either standard deviation, significance values, or CIs. Standard deviation was computed for all significance values or CIs. These data were pooled and a meta-analysis performed for the continuous data. Mean differences in the mean age at incidences between TNBC and non-TNBC for the 12 studies were plotted in a forest plot with both the random-effects and the fixed-effects models because the heterogeneity (I^2) was close to 50%.

For meta-analysis of categorical data, namely binned age at incidence, high grade (grade 3), and lymph node positivity within TNBC and non-TNBC cohorts, pooled odds ratios (ORs) of the binary outcomes were estimated. When the heterogeneity index (I^2) was $> 50\%$ and/or significant ($P < .05$), the DerSimonian-Laird method was used for the pooled estimate of the ORs using the random-effects model. Otherwise, the Mantel-Haenszel method was used to obtain the fixed-effects model of the pooled ORs. Data were analyzed using the meta package in R.[61] Study outliers are assessed by visual inspection of the funnel plot for OR against SE. Data were re-analyzed and forest plots replotted after removal of the outliers to examine outlier effect.

RESULTS

To assess the variability and the source of variability in the prevalence rates of TNBC in Indian patients with breast

cancer, we selected and curated studies in breast cancer cohorts of Indian and/or Indian-origin patients (Fig 1). A systematic review of 34 such research articles published during 2009-2019 that reported incidence numbers and clinical parameters of patients with breast cancer according to molecular subtype is compiled in the Data Supplement. The average cohort size was 608 patients (range, 72-5,436 patients), with a total number of 20,678 patients. The meta-analysis was performed for prevalence of TNBC. Clinical parameters associated with the aggressive characteristic of the disease, such as age at incidence, high grade, and lymph node positivity, were compared between TNBC and non-TNBC using summary OR with 95% CIs.

Prevalence of TNBC Within Indian Cohorts

Pooled prevalence of TNBC for all 34 studies was 27% (95% CI, 24% to 31%; Fig 2). The lowest prevalence observed was 7%[12] and 11.8%,[62] while the highest prevalence was 47%[14] and 50%[40] (Fig 2). Thus, significantly high heterogeneity was observed among the 34 studies (I^2 = 95.1%), with > 50% of the studies falling outside the CIs (Data Supplement).

In an attempt to understand the source of heterogeneity, we tested whether different ER expression cutoffs used to define ER positivity had any influence on the TNBC prevalence. Accordingly, the 34 studies were grouped into 3 subgroups. Heterogeneity analysis was computed for TNBC prevalence for each subgroup independently (Data Supplement). All 3 subgroups had high heterogeneity. The highest heterogeneity was observed for subgroup 3, where the studies did not mention a cutoff for ER/PR positivity. Combined prevalence was computed for each subgroup using a random-effects model. Subgroup 1, which used 1% ER expression as cutoff, showed a higher prevalence of TNBC (30%; 95% CI, 26% to 34%) compared with subgroup 2, which used 5%-10% ER expression as the cutoff (24%; 95% CI, 19% to 30%; Fig 2). Subgroup 3 (no mention of cutoff) showed a prevalence of 26% (95% CI, 21% to 33%). Among subgroups, prevalence variation was not significant, as revealed by the test of moderators, indicating that the heterogeneity within subgroups as well as in all 34 studies may be due to factors other than ER expression cutoff used.

Age at Incidence

Twenty of the 34 studies reported age at incidence for patients with and without TNBC in the cohort. Mean age at incidence for TNBC was 47.52 ± 3 years, which is significantly younger than that for non-TNBC (51.02 ± 2.4; P = 0.005; n = 12; Data Supplement). In 9 of 12 studies, mean difference in the mean age at incidence between TNBC and non-TNBC was plotted as a continuous forest plot using a fixed-effects model. A weighted mean difference of –2.75 (95% CI, –3.59 to –1.92) significantly favored younger age at incidence for TNBC (Fig 3A). The studies were highly homogeneous, as observed in the funnel plot shown in Data Supplement.

For the studies that reported age at incidence in bins of < 50 years and > 50 years, the ORs for incidence at < 50 years were computed and plotted as a dichotomous forest plot (n = 8). Study heterogeneity was observed to be 55%; hence, both random- and fixed-effects models were used. Both models showed significantly higher odds for age < 50 years for incidence of TNBC compared with non-TNBC (pooled OR, 1.35; 95% CI, 1.08 to 1.69) in the random-effects model (Fig 3B). The studies are more or less homogeneous as observed in the funnel plot (Data Supplement). Either as mean age or grouped age, TNBC in Indian cohorts seem to consistently present at a significantly younger age than non-TNBC.

Tumor Grade

Higher histologic grade of tumor at incidence is a significant predictor of poor prognosis. Patients with TNBC were more likely to have higher histologic tumor grade than those without TNBC.[63,64] We compared grade records for TNBC versus non-TNBC within the Indian cohorts. Fourteen of the 34 studies had grade records for patients with and without TNBC. We compared the ORs for the proportion of grade 3 within TNBC with respect to grade 3 within non-TNBC (Fig 4A). The heterogeneity analysis using funnel plot was significantly high with 3 outliers (Data Supplement). The outliers were removed and ORs pooled and re-analyzed for the 11 studies. Heterogeneity within the 11 studies was < 50%; hence, the forest plot for ORs was plotted using the fixed-effects model. For these 11 studies, patients with TNBC had a 2.57 times higher odds of presenting with high-grade (grade 3) disease compared with those without TNBC, with a highly significant overall effect (Fig 4B).

Lymph Node Positivity

Tumor metastasis to axillary lymph nodes is one of the prognostic factors for breast cancer recurrence.[65] We analyzed Indian cohorts for any difference in proportions of lymph node positivity between TNBC and non-TNBC at presentation. Fourteen of 34 studies reported data on lymph node status for patients with and without TNBC. The pooled data for lymph node positivity in TNBC and non-TNBC showed high heterogeneity and were therefore analyzed by random-effects model. The pooled OR favored lymph node positivity in TNBC (OR, 1.35; 95% CI, 0.94 to 1.92), but the overall effect was not significant (Fig 5A). Visual inspection of the funnel plot for these data showed 3 outliers.[18,31,37] Re-analysis of the pooled data from the remaining 11 studies after removal of the outliers showed a marked decrease in heterogeneity. The pooled OR showed that lymph node positivity was favored in TNBC over non-TNBC (OR, 1.39; 95% CI, 1.21 to 1.60), with a significant overall effect (Fig 5B).

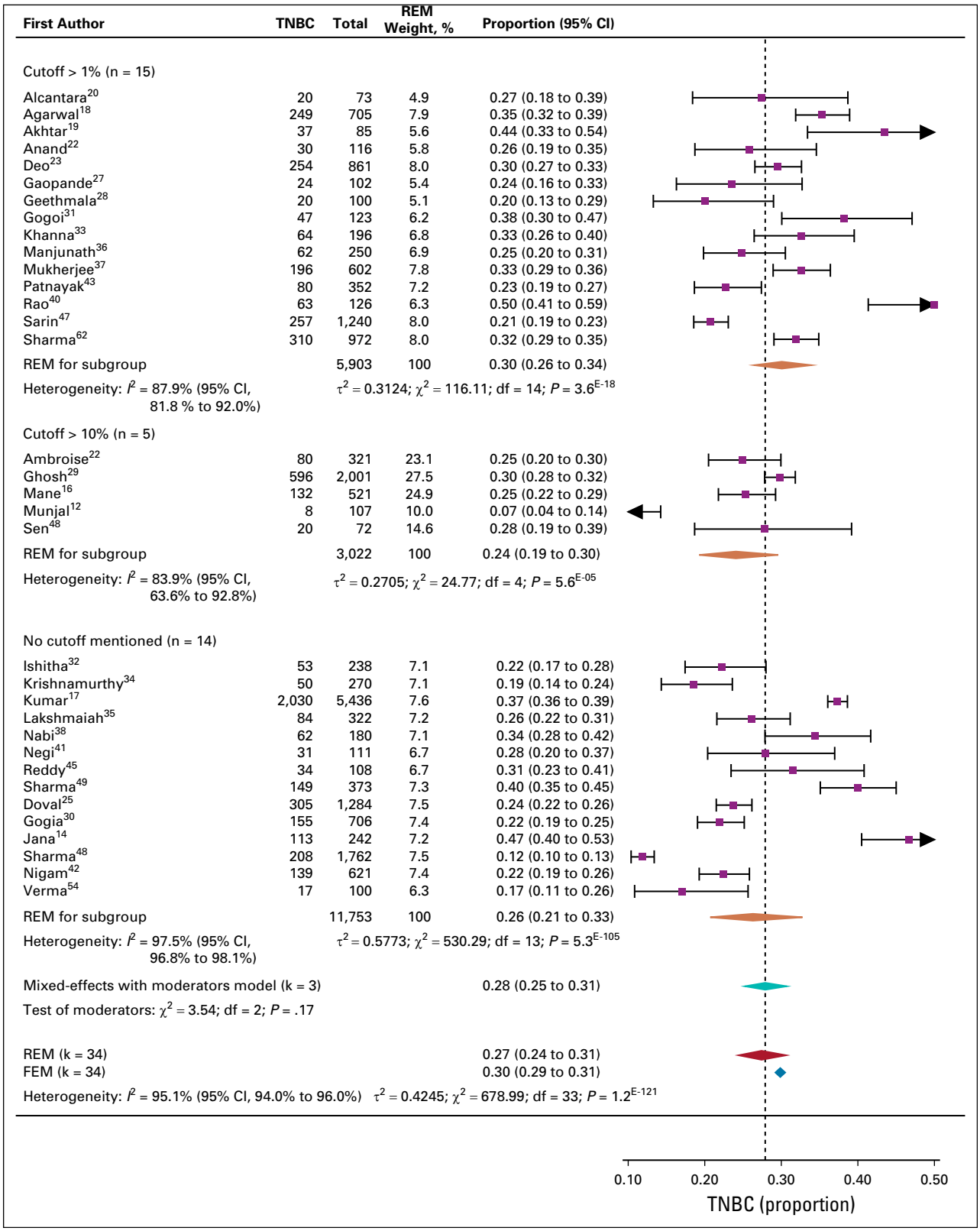


FIG 2. Prevalence of triple-negative breast cancer (TNBC) in Indian cohorts. Forest plot of prevalence (%) of TNBC within 34 studies that reported data on Indian patients with breast cancer. Subgroups were made by guidelines followed for estrogen receptor (ER)/progesterone receptor (PR) positivity: studies that used 1% expression of ER/PR as a cutoff, 10% expression of ER/PR as a cutoff, or studies that did not mention the ER/PR expression criteria. Heterogeneity (I^2) is noted to be significantly high for each subgroup as well as for all 34 studies together. Combined pooled prevalence for all 34 studies estimated by a random-effects model (REM) is shown in purple. The fixed-effects (continued on following page)

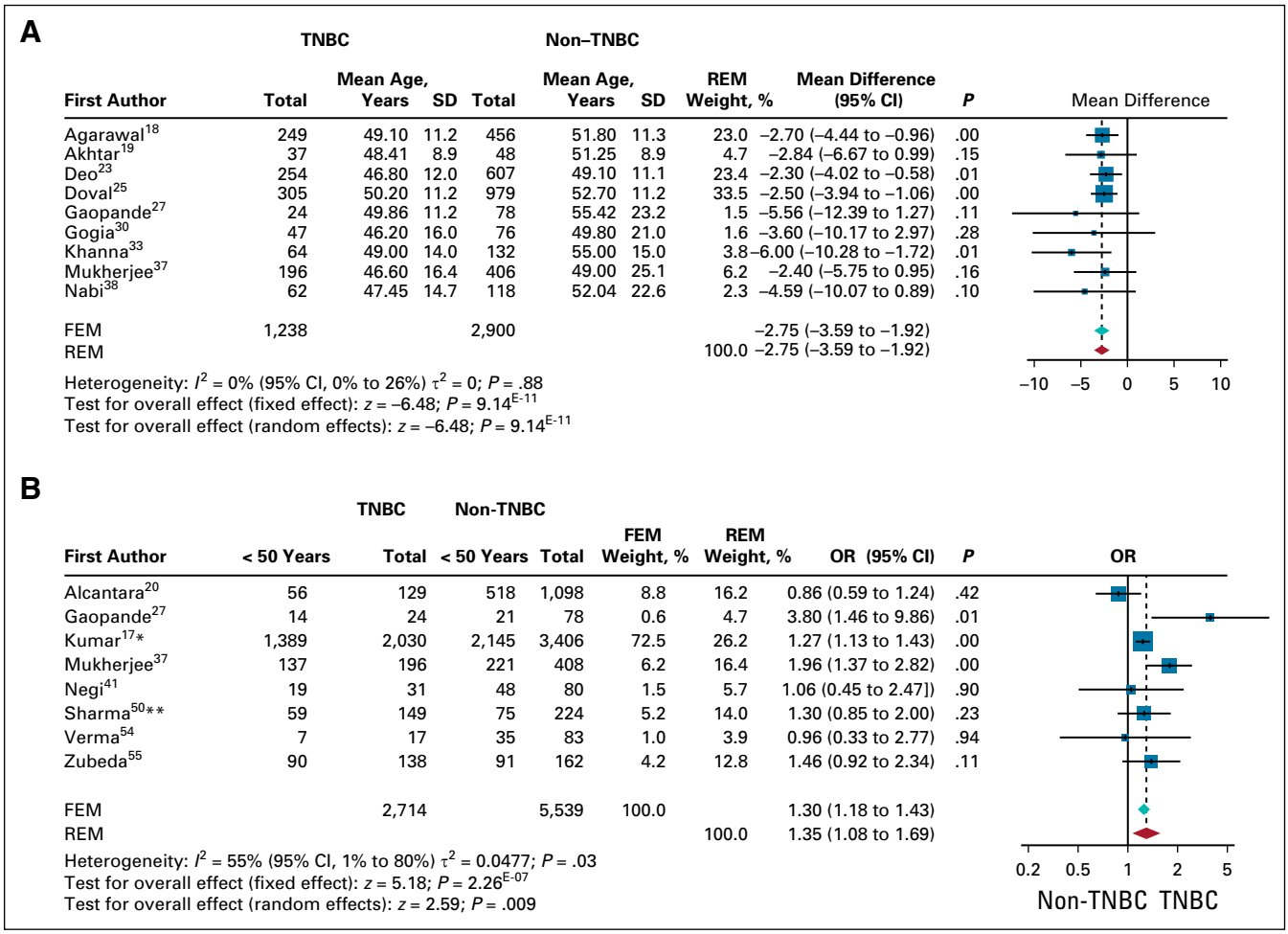


FIG 3. Age at incidence compared for triple-negative breast cancer (TNBC) and non-TNBC. (A) Forest plot for weighted mean difference in mean age at incidence between TNBC and non-TNBC for 9 studies. Studies were highly homogenous, with significant odds for younger age at incidence in patients with TNBC. The *P* value for comparison within each study is also shown. The red diamond and dotted line represent the random-effects pooled estimate of the weighted mean difference in age at incidence between patients with and without TNBC, while the teal diamond represents the fixed-effects model (FEM). (B) Binned age at incidence. The pooled odds ratio (OR) is computed by both FEM and random-effects model (REM) because the heterogeneity among 8 studies was found to be close to 50%. The pooled OR of the FEM is represented by a teal diamond, and the REM is represented by a red diamond. The dotted line represents the OR of the REM. OR of individual studies are represented by blue boxes. The boxes are weighted by the study weights in the REM. The *P* value for each study is mentioned. (*)The bins represent a cutoff of 55 years instead of 50 years. (**)Cutoff of 40 years. SD, standard deviation.

DISCUSSION

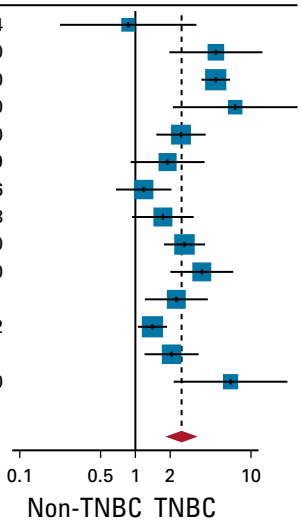
To our knowledge, this systematic review of 34 studies is one of a kind, covering the highest number of peer-reviewed articles published so far for Indian patients with breast cancer, and for the first time, a possible source of heterogeneity observed in the prevalence of TNBC and OR of clinical features at presentation within Indian cohorts has been systematically explored. Pooled prevalence from all 34 studies was found to be high at 27% (95% CI, 24% to 31%), with high variation (range, 7%-50%). The reviews published in an earlier report indicated similar variation, although with a fewer number of articles.[7,8] We reason that the differences in defining ER positivity may have contributed to the variation in prevalence. Revised ASCO-CAP guidelines for ER/PR positivity were published in 2010, yet we did encounter studies published as late as 2015[16] that followed the 10% ER expression cutoff as a guideline to call out ER-positive breast cancer. Half of the studies did not

FIG 2. (Continued). estimate is also shown. Pooled prevalence for each subgroup computed with random effects is shown as orange diamonds. The subgroup variances are pooled together in a mixed-effects model (FEM; teal diamond). Intergroup variance in pooled prevalence is tested by Wald test, and test of moderators are not significant. The spread of each diamond represents the 95% CIs for the pooled estimate. The dotted line represents the proportion calculated from the mixed-effects model with moderators.

A

First Author	TNBC Grade 3	TNBC Total	Non-TNBC Grade 3	Non-TNBC Total	REM Weight, %	OR (95% CI)	P
Alcantara[20]	17	20	85	98	3.6	0.87 (0.22 to 3.37)	.84
Akhtar[19]	24	37	13	48	5.6	4.97 (1.97 to 12.57)	.00
Doval[25]	223	305	347	979	9.7	4.95 (3.72 to 6.59)	.00
Gaopande[27]	8	24	5	78	4.1	7.30 (2.11 to 25.26)	.00
Gogia[30]	39	89	77	322	8.4	2.48 (1.52 to 4.05)	.00
Gogoi[31]	26	47	30	76	6.8	1.90 (0.91 to 3.96)	.09
Jana[14]	81	169	32	73	8.0	1.18 (0.68 to 2.05)	.56
Khanna[33]	41	64	67	132	7.6	1.73 (0.94 to 3.20)	.08
Mane[16]	65	132	104	389	9.0	2.66 (1.77 to 4.00)	.00
Mukherjee[37]	140	153	218	294	7.5	3.75 (2.01 to 7.02)	.00
Nabi[38]	35	62	43	118	7.5	2.26 (1.21 to 4.23)	.01
Sharma[62]	201	297	380	635	9.7	1.41 (1.05 to 1.88)	.02
Sharma[50]	42	91	45	153	8.1	2.06 (1.20 to 3.53)	.01
Verma[54]	9	17	12	83	4.6	6.66 (2.15 to 20.65)	.00
REM		1,507		3,478	100.0	2.51 (1.82 to 3.46)	

Heterogeneity: I^2 = 78% (95% CI, 63% to 87%) τ^2 = 0.2566; P < .01
Test for overall effect: z = 5.61; P = 1.96^{E-08}



B

First Author	TNBC Grade 3	TNBC Total	Non-TNBC Grade 3	Non-TNBC Total	FEM Weight, %	OR (95% CI)	P
Alcantara[20]	17	20	85	98	3.4	0.87 (0.22 to 3.37)	.84
Akhtar[19]	24	37	13	48	3.1	4.97 (1.97 to 12.57)	.00
Gaopande[27]	8	24	5	78	1.2	7.30 (2.11 to 25.26)	.00
Gogia[30]	39	89	77	322	14.8	2.48 (1.52 to 4.05)	.00
Gogoi[31]	26	47	30	76	8.1	1.90 (0.91 to 3.96)	.09
Khanna[33]	41	64	67	132	12.4	1.73 (0.94 to 3.20)	.08
Mane[16]	65	132	104	389	21.1	2.66 (1.77 to 4.00)	.00
Mukherjee[37]	140	153	218	294	10.0	3.75 (2.01 to 7.02)	.00
Nabi[38]	35	62	43	118	10.2	2.26 (1.21 to 4.23)	.01
Sharma[50]	42	91	45	153	14.2	2.06 (1.20 to 3.53)	.01
Verma[54]	9	17	12	83	1.5	6.66 (2.15 to 20.65)	.00
FEM		736		1,791	100.0	2.57 (2.12 to 3.12)	

Heterogeneity: I^2 = 30% (95% CI, 0% to 66%) τ^2 = 0.0496; P = .16
Test for overall effect: z = 9.52; P = 1.79^{E-21}

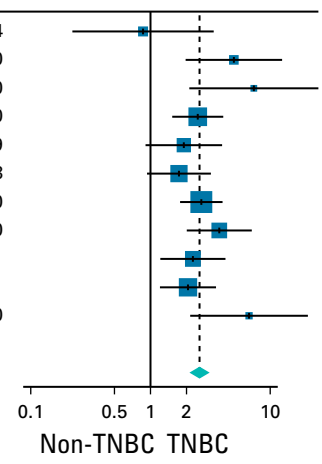


FIG 4. Comparison of high grade at incidence between triple-negative breast cancer (TNBC) and non-TNBC. (A) Forest plot of odds ratio (OR) for high grade in TNBC *v* non-TNBC for 14 studies. Studies were significantly heterogenous and showed higher ORs for high grade at incidence in TNBC. The *P* value for comparison within each study is also shown. The red diamond and the dotted line represent the random effects pooled estimate of the OR, while the teal diamond represents the fixed-effects model (FEM). (B) Forest plot of ORs for high grade after removal of outliers. The analysis shows 3 studies (Doval et al,[25] Sharma et al,[49] and Jana et al[14]) to be outliers in the pooled OR (Data Supplement). These studies were excluded and the pooled OR recomputed. Because the heterogeneity among the remaining the 10 studies is reduced (I^2 = 30%), the fixed-effects estimate of the pooled OR is shown (green diamond). The pooled OR shows a significantly increased OR for high grade in TNBC *v* non-TNBC. FEM, fixed-effects model; REM, random-effects model.

mention the cutoff they used to define ER positivity. Irrespective of the guidelines followed, all the subgroups presented with high heterogeneity as well as variation in TNBC prevalence.

Thirty percent prevalence in the subgroup with 1% cutoff for ER/PR positivity is alarmingly high, as pointed out earlier.[8] Significantly high heterogeneity within this subgroup may indicate inconsistent IHC diagnostic methods. It is highly likely that factors like uneven tissue fixation, inadequate retrieval methods, and use of different and/or unconventional antibodies contributed to lower expression of ER and hence, high TNBC prevalence (reviewed in Shet[9]). Similar to ER-positive selection criteria, some of the studies used HER2 score with no reference to

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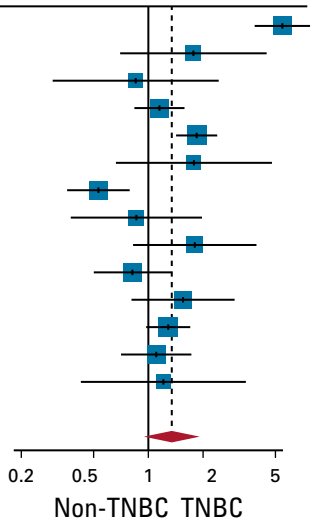
First Author	TNBC LN Positive	TNBC Total	Non-TNBC LN Positive	Non-TNBC Total	REM Weight, %	OR (95% CI)	P
Agarwal[18]	192	249	174	456	8.5	5.46 (3.84 to 7.75)	.00
Akhtar[19]	27	37	29	48	5.7	1.77 (0.70 to 4.47)	.23
Alcantara[20]	10	19	30	53	5.1	0.85 (0.30 to 2.44)	.77
Deo[23]	178	254	407	607	8.6	1.15 (0.84 to 1.58)	.39
Doval[25]	179	305	426	979	8.9	1.84 (1.42 to 2.39)	.00
Gaopande[27]	17	24	45	78	5.4	1.78 (0.66 to 4.78)	.25
Gogia[30]	69	130	313	460	8.3	0.53 (0.36 to 0.79)	.00
Jana[14]	92	104	116	129	6.1	0.86 (0.37 to 1.97)	.72
Khanna[33]	54	64	99	132	6.4	1.80 (0.82 to 3.93)	.14
Mukherjee[37]	62	101	144	218	7.9	0.82 (0.50 to 1.33)	.42
Nabi[38]	43	62	70	118	7.1	1.55 (0.81 to 2.98)	.19
Sharma[62]	173	294	338	642	8.8	1.29 (0.97 to 1.70)	.08
Sharma[50]	103	149	150	224	8.1	1.10 (0.71 to 1.72)	.66
Verma[54]	9	17	40	83	5.1	1.21 (0.43 to 3.44)	.72
REM		1,809		4,227	100.0	1.35 (0.94 to 1.92)	

Heterogeneity: I^2 = 86% (95% CI, 79% to 91%) τ^2 = 0.3497; P < .01
Test for overall effect: z = 1.65; P = .10



B

First Author	TNBC LN Positive	TNBC Total	Non-TNBC LN Positive	Non-TNBC Total	FEM Weight, %	OR (95% CI)	P
Akhtar[19]	27	37	29	48	2.0	1.77 (0.70 to 4.47)	.23
Alcantara[20]	10	19	30	53	2.2	0.85 (0.30 to 2.44)	.77
Deo[23]	178	254	407	607	20.9	1.15 (0.84 to 1.58)	.39
Doval[25]	179	305	426	979	24.3	1.84 (1.42 to 2.39)	.00
Gaopande[27]	17	24	45	78	1.8	1.78 (0.66 to 4.78)	.25
Jana[14]	92	104	116	129	3.5	0.86 (0.37 to 1.97)	.72
Khanna[33]	54	64	99	132	2.9	1.80 (0.82 to 3.93)	.14
Nabi[38]	43	62	70	118	4.3	1.55 (0.81 to 2.98)	.19
Sharma[62]	173	294	338	642	25.4	1.29 (0.97 to 1.70)	.08
Sharma[50]	103	149	150	224	10.8	1.10 (0.71 to 1.72)	.66
Verma[54]	9	17	40	83	1.9	1.21 (0.43 to 3.44)	.72
FEM		1,329		3,093	100.0	1.39 (1.21 to 1.60)	

Heterogeneity: I^2 = 4% (95 CI, 0% to 62%) τ^2 = 0.0026; P = .40
Test for overall effect: z = 4.70; P = 2.664 x 10^6

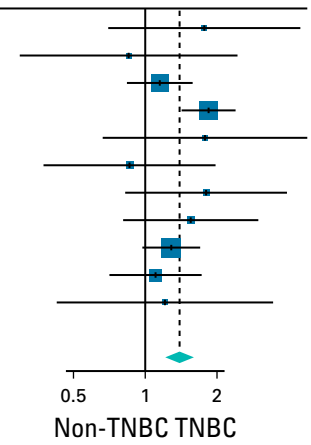


FIG 5. Comparison of lymph node (LN) positivity at incidence between triple-negative breast cancer (TNBC) and non-TNBC. (A) Forest plot of odds ratios (ORs) of LN positivity between TNBC and non-TNBC in 14 studies. Studies were heterogenous, and increased ORs for LN positivity in TNBC were not significant except for Doval et al.[25] The pooled OR is also not significant. The *P* value for comparison within each study is also shown. The red diamond and dotted line represent the random effects pooled estimate of the OR. (B) Comparison of LN-positive incidence after removal of outliers. Funnel plot analysis shows that 3 studies (Gogoi et al,[31] Mukherjee et al,[37] and Agarwal et al[18]) were outliers of the pooled OR (Data Supplement). These studies were excluded and the pooled OR recomputed. Because the heterogeneity among the remaining 11 studies is low (I^2 = 4%), the fixed-effects estimate of the pooled OR was computed (teal diamond). The overall effect without the outliers shows a significantly higher OR for LN positivity in TNBC *v* non-TNBC. FEM, fixed-effects model; REM, random-effects model.

FISH. Definitions for both ER/PR and HER2 positivity are determining factors in defining TNBC. Inconsistent methods as well as variation in detection methods across centers may have contributed to false-negative reporting of ER/PR and HER2 expression, especially for tumors that inherently express these receptors at low levels. Of the 34 studies reviewed here, 1 reported that with the improvement in the detection methods and protocol over a period of 6 years, the percentage of patients with TNBC reduced from 40% to 26% in its cohort.[17]

Our meta-analysis reveals that the variations in tissue processing and detection protocols and/or standards as well the lack of reporting thereof (eg, subgroup 3 in our analysis) might be important and major contributing factors

toward the observed variation in TNBC prevalence in Indian cohorts. An analysis of SEER data in the United States after 1988 showed that women of Asian Indian/Pakistani origin had significantly higher rates of ER/PR-negative cancer than White women (30.6% *v* 21.8%; $P = .0095$).[66] This cohort may be considered as representative of a population of Indian immigrants and their descendants in the United States.[67] Because this data set did not include HER2 status, ER/PR-negative cancers represent both HER2 and TNBC. This cohort was found to be significantly younger (< 50 years of age) and presented with higher-grade cancer. More recently, Plasilova et al[6] analyzed updated data from SEER, which included HER2 by IHC and FISH. In detailed race analysis of cancer subtypes, the authors reported subtype data for Asian Indians. The proportion of TNBC in the Asian Indian group (15.4%) was found to be significantly higher than in the White group (11.6%; $P = .0003$), and the second highest among all the races reported. Although significantly higher, the TNBC incidence rate for the Asian Indian population (15.6%) is substantially lower than the pooled TNBC prevalence of 27% shown in Figure 2. Even with greater standardization of diagnostic procedures and reporting, the TNBC rate in a biased Indian-origin subpopulation is high compared with White rates. This reinforces the conclusion that TNBC rates in an Indian/Indian-origin population are intrinsically high; however, some of the observed increase in our meta-analysis may stem from diagnostic limitations across the country.

With this backdrop, it will be interesting to systematically review the IHC protocols used in Indian centers as a source of variation. High TNBC in Indian cohorts may be partly due to a lack of standardized protocols and stringent guidelines in diagnostic centers leading to inadequate assessment of hormone receptor expression. Moving forward, standardized protocols with unified and stringent guidelines are essential in the Indian setting for accurate assessment of hormone receptor expression in breast cancers. In the meantime, performance of PAM50 on select and representative cohorts may give a true assessment of TNBC proportions in Indian cohorts.[68] The difference between the biologically high TNBC prevalence and the observed TNBC prevalence may become clearer as health care standards improve across the country.

Regardless of high prevalence and variation in the prevalence, the clinical features that TNBC presents within Indian cohorts tend to be aggressive compared with non-TNBC, similar to the observations in western cohorts.[6] All the studies reviewed here reported that patients with TNBC are significantly younger and that significantly higher proportions have high-grade tumors compared with non-TNBC (except for Alcantara et al[20]), with studies being highly homogeneous. For lymph node positivity, heterogeneity was low (after removal of outliers), and all the studies except 2 (Alcantara et al and Jana et al[14]) showed higher odds for TNBC to present with lymph node positivity. The pooled OR is significantly in favor of TNBC after the removal of outliers.

In summary, the systematic review of Indian cohorts once again reflects a higher prevalence of TNBC within Indian cohorts. Indian patients with TNBC presented at a younger age compared with those without TNBC and with a significantly higher proportion of aggressive clinical features, such as high grade and lymph node positivity. In India, TNBC with its higher prevalence and clinically aggressive features needs focus and consistent efforts to identify an appropriate and effective treatment regimen to tackle this clinically challenging disease. Before that, however, India needs to standardize the detection methods that identify this aggressive subtype of breast cancer with accuracy.

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Diagnostic Pathology

RESEARCH

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Evaluation of tumor-infilt ating lymphocytes (TILs) in molecular subtypes of an Indian cohort of breast cancer patients

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Abstract

Objectives: Evaluation of tumor-infiltrating lymphocytes (TILs) distribution in an Indian cohort of breast cancer patients for its prognostic significance.

Methods: A retrospective cohort of breast cancer patients from a single onco-surgeon's breast cancer clinic with a uniform treatment strategy was evaluated for TILs. Tumor sections were H&E stained and scored for the spatial distribution and percent stromal TILs infiltration by a certified pathologist. The scores were analysed for association with treatment response and survival outcomes across molecular subtypes.

Results: Total 229 breast cancer tumors were evaluated. Within spatial distribution categories, intra-tumoral TILs were observed to be associated with complete pathological response and lower recurrence frequency for the entire cohort. Subtype-wise analysis of stromal TILs (sTILs) re-enforced significantly higher infiltration in TNBC compared to HER2-positive and ER-positive tumors. A favourable association of higher stromal infiltration was observed with treatment response and disease outcomes, specifically in TNBC.

Conclusion: Intra-tumoral TILs showed a higher proportion with favourable association with better patient outcomes in an Indian cohort, unlike western cohorts where both stromal and intra-tumoral TILs show similar association with prognosis. With further validation, TILs can be developed as a cost-effective surrogate marker for treatment response, especially in a low-resource setting such as India.

Keywords: TILs, TNBC, Breast Cancer in India

Key points

Key point 1:

The study is one of the first comprehensive evaluations of tumor-infiltrating lymphocytes in an Indian cohort of breast cancer patients.

Key point 2:

Intra-tumoral TILs presented in a higher proportion of patients, specifically in the TNBC subtype in an Indian cohort.

Key point 3:

Stromal TILs infiltration showed a higher distribution of TILs infiltration across TNBC tumors, where higher scores co-related with better therapy response and longer disease-free survival.

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Introduction

Breast Cancer is the leading cause of cancer-related female deaths in India, with close to a 50% mortality rate [1]. Though there are numerous targeted treatments are available for molecular subtypes with hormone receptor expression: ER, PR and/or HER2, as of now there is only targeted therapy available for TNBC which is directed towards immune response modulators [2]. Furthermore, TNBC is an aggressive subset of breast cancer with unpredictable response to therapy and hence higher rates of recurrence and lower overall survival [3, 4].

Worldwide, the prevalence of TNBC is 10–12% [5, 6], while in India, the prevalence of TNBC is reported to be significantly higher and up to 20–30% [7–9]. A greater proportion of TNBC cases in an Indian cohort are presented with aggressive clinicopathological features such as younger age, premenopausal status, and tumor with high grade [7]. With no targetable treatment and higher proportions of aggressive disease at incidence, TNBC poses a clinical management challenge in India, especially with a high proportion of incidences at a younger age.

The standard treatment for TNBC includes surgery for lymph node-negative patients and neoadjuvant chemotherapy (NACT) for patients with node metastasis, followed by surgery [10–12]. As of now, the treatment strategy of TNBC is determined by clinicopathologic features such as tumor size, proliferative index, lymph node involvement as well as a pathological response to chemotherapy in the neoadjuvant setting. TNBC is reported to show a better response to NACT compared to other subtypes, with 22 to 56% patients reported with pathologically complete response depending on the treatment regime [3, 13]. Pathological complete response in the TNBC subtype is shown to be associated with better disease-free survival [13]. Cases with the residual disease have been shown to have a significantly higher chance of recurrence within 1st three years of treatment and reduced overall survival [14, 15].

Recent studies have revealed tumor infiltrating lymphocytes (TILs) to be a promising predictive biomarker for therapy response, especially in TNBC [16–18]. Tumor infiltrating lymphocytes are cytotoxic lymphocytes infiltrating into the tumor and stromal regions as a host immune response [19, 20]. A greater extent of infiltration of lymphocytes, especially in the tumor stroma, enhances the anti-tumor effects of the therapy [21, 22]. Meta-analysis of 3770 patients with higher TILs scores associated with complete response to NACT in 50% of the TNBC patients, which was further associated with better long-term survival over three years, emphasizing the prognostic significance of TILs in TNBC [23, 24].

With a high incidence rate of TNBC in India, understanding TILs distribution with respect to treatment response and survival outcomes may help develop TILs as an accessible biomarker to predict treatment response in TNBC. TILs evaluation may provide a promise to predict a responsive subset of TNBC in India and help to de-escalate further chemotherapy.

Here we evaluate TILs with respect to clinicopathological features, treatment response and survival outcomes of a breast cancer patient cohort from a single surgeon and oncologist breast cancer unit in India [25].

Materials and methods

Patient tissue samples and meta-data

Primary breast tumor tissue (Formalin-fixed paraffin-embedded, FFPE) samples and associated de-identified patient metadata was received from the biobank [25], with appropriate patient consent and ethical approval (dated 21st July 2018 #IECHR/VB/2018/016). Patients who were diagnosed and underwent treatment from 2012 up to 15th July 2021 are included in the study cohort. Patient data, including diagnostic clinical and pathological reports, treatment regimens and post-treatment follow-up data up to last follow-up /recurrence date/death, was curated and digitized. All FFPE tissue blocks used were of primary (pre-treatment) tumor tissue. Of the 229 FFPE tumor samples, 179 were derived from true-cut core biopsy, and fifty were derived from naive tumor tissue excised during surgery.

Molecular subtypes of the breast tumors were determined based on immunohistochemistry and FISH reports from an accredited pathology lab. Samples were categorized into ER-positive, HER-positive, and TNBC subtypes based on ER/PR expression and HER2 scores. ER+ tumor samples were identified to be with more than 1% ER expression as positive for ER, HER2 IHC expression score of 0, 1+ or 2+ with FISH-negative report as negative for HER2 and positive or negative PR expression. Samples with HER2 IHC score of 3+ or score of 2+ with positive FISH and negative for ER expression with less than 1% expression, irrespective of PR expression were marked as HER2-positive subtype. Triple-negative samples were defined as the ones with less than 1% ER and less than 1% PR expression with HER2 IHC expression scores of 0, 1+ or 2+ and FISH-negative report.

NACT and ACT treatment was administered as per NCCN guidelines. The standard treatment for breast cancer patients with negative lymph nodes is surgery followed by ACT (adjuvant chemotherapy). For patients with lymph-node positivity, the standard treatment is NACT (neo-adjuvant chemotherapy), followed by surgery and ACT, and, if required, radiation therapy. TNBC patients ($n=26$) were treated with Taxanes with

or without Anthracycline/Cyclophosphamide (AC) or 5-Fluoro-Uracil/AC regimen as NACT and/or ACT regimen wherever appropriate. ER+ patients ($n=33$) were treated with anti-estrogens such as Letrozole or Tamoxifen in case of NACT and AC + taxane regimen in case of ACT. For HER2 positive patients ($n=17$), AC followed by Taxane with Trastuzumab was administered as NACT and Trastuzumab with or without a Taxane as NACT, and/or FAC and Taxane as ACT.

Treatment response

Response to NACT was computed for 60 out of 76 patients who underwent NACT for whom clinical tumor size (cT) and node status (cN), as well as post-NACT tumor size (ypT) and node status (ypN), was available. Post-NACT, response to treatment was calculated by comparing cTcN values with ypTyPN. In case of residual DCIS or absence of residual tumor and absence of lymph node metastasis during pathological examination of surgically removed tissue, i.e., ypTisN0/ ypT0N0 status, the response is considered as pathological Complete Response (pCR). The presence of a residual tumor and/or lymph node metastasis in surgically removed tissue is referred to as Residual Disease (RD).

Histopathology of FFPE tissue blocks

FFPE blocks of the primary tumor were processed for histopathology. Tumor sections of 4–5 µm were obtained using Leica Microtome RM2255. Tissue slides were deparaffinized. Each slide was cleaned and stained with a drop of undiluted Hematoxylin solution (Delafield, 38,803) in a humidifying chamber for 15 mins, followed by 1% eosin (Qualigen Q39312). The slides were then gradually dehydrated in ethanol solutions followed by Xylene. Slides were mounted in DPX (Q18404).

Imaging of histopathology slides

All slides were imaged by OptraScan using the OS-15 bright field digital scanner at 400X magnification. Images were viewed using the 'Image viewer' software provided by Optra. The images were then converted to TIFF format and processed using Image Viewer Version 2.0.4 by OptraScan for scale bars.

Spatial TILs scoring

Tumor-infiltrating lymphocytes (TILs) distribution in the tumor microenvironment was assessed from H&E sections. The spatial distribution of TILs was estimated based on the proximity of lymphocytes to the tumor cells, i.e. intra-tumoral TILs (I): TILs present within the tumor core and adjacent to tumor cells, peri-tumoral TILs (P): TILs present in the periphery of the tumor core, but restricted to surrounding stroma, stromal TILs (S): TILs present in the stromal tissue with no significant proximity to the tumor core, and desert TILs (D): TILs absent from both the tumor core as well as the stroma. For tumor samples with more than one type of spatial phenotype, a single score was taken based on the predominant presentation. Spatial distribution was scored twice independently with 100% concordance.

Percent stromal tumor-infiltrating lymphocytes (sTILs) scoring

Percent TILs distribution of sTILs within stroma surrounding the tumor tissue was assessed from the H&E-stained histopathology section of primary tumor tissue. The scoring was done by the pathologist according to the recommendations of The TILs Working group [22]. The pathologist was blinded to the clinical data as well as molecular subtype information.

Post-NACT sTILs scoring

For patients treated with NACT ($n=76$), sTILs scores from post-NACT surgery samples were procured from the pathologist's report. For patients with no report for sTILs scores, surgery H&E slides created at the time of original pathology were retrieved from storage and scored by the pathologists for sTILs.

Response to treatment analysis

Response to Neo-adjuvant Chemotherapy (NACT) was calculated by comparing clinical tumor size (cT) and lymph node status (cN) to pathological tumor size (ypT) and pathological lymph node status (ypN). Post-NACT pathological status if yPT0 (no residual tumor) or ypTis (residual DCIS) and no lymph node involvement (ypN0), the response was defined as pathological Complete Response (pCR). For patients with residual tumor (ypT1-ypT4) and/or lymph node metastasis (ypN1–3) in a post-NACT setting, the response was noted as Residual Disease (RD).

Response to NACT across the subtypes is then analyzed using a 3×4 Chi-square contingency test. Box plot for percent sTILs scores according to the response to NACT, i.e., pCR and RD, is plotted using GraphPad Prism v.5. Mean sTILs with S.E. according to the response to NACT for each subtype is computed and plotted by using GraphPad Prism v.5.

sTILs scores of primary biopsy and post-treatment surgery tissues were plotted using a before-after graph using GraphPad Prism v.5. Paired t-test was performed to assess the significance of the difference in mean sTILs scores between primary and post-NACT tumor tissue.

Statistical analysis

All statistical analysis was done using GraphPad Prism v.5. A demographic table was prepared using IBM SPSS Statistics v. 21.0.0.0. The distribution of clinicopathological characteristics within the cohort and breast cancer subtypes was analyzed using a 2×3 (4×3 in case of tumor size) Chi-square contingency test. Mean age across subtypes was compared using the Mann Whitney test. Column statistics for sTILs were computed on GraphPad Prism v.5 to calculate mean and standard error across each sub-category of clinical characteristics. A significant difference in distribution across ER+, HER2+ and TNBC sTILs scores for clinicopathological characteristics were analyzed with the Kruskal-Wallis test. Individual comparison across two groups was analyzed by the Mann-Whitney test. All graphs were plotted using GraphPad Prism v.5.

Survival outcome analysis

Survival outcomes were computed as follows: disease-free survival (DFS) was calculated as time in months from the date of surgery till the date of recurrence or last follow-up date. Overall survival (OS) was calculated as time in months from the diagnosis date (biopsy date) till the last follow-up date or date of death due to disease. Kaplan-Meier survival plots for DFS and OS for up to 5-years follow-up time were plotted, and survival probabilities were computed by Log-rank, Breslow, and Tarone-Ware cores towards 5-years DFS and OS using IBM SPSS Statistics v 21.0.0.0.

Results

Breast cancer cohort characteristics

Primary tumor tissue samples of IDC patients were identified and retrieved for 229 cases from the breast cancer biobank. Demographic and clinical characteristics of the cohort are presented according to their molecular subtypes (ER+, HER2+ and TNBC) in Table 1. The average age of the cohort of 229 breast cancer patients is 54.4, ranging from 28 to 86 years. TNBC patients ($n=81$) presented with a significantly higher proportion of younger age (mean age of 51.8 ± 12 years) and premenopausal patients (39.4%) compared to ER+ and HER2+ patients (mean age of 56.1 ± 12 and 55.1 ± 12 years; respectively) with 28.0 and 21.6% premenopausal cases. TNBC reflected a significantly higher proportion of high-grade tumors (76.5% grade III) compared to other subtypes (25.6 and 56.9%, respectively). Clinical and pathological tumor size, lymph node positivity, stage and LVI did not differ significantly across all three subtypes. Overall and disease-free survival data were available for 219 and 197 patients, respectively. The cohort had an average follow-up of 22 months and a median follow-up of 14 months.

Out of 229 patients, 76 (34.9%) received neoadjuvant chemotherapy (NACT) according to their clinical and hormone receptor expression status as described in the methods section. Of these 76, 15 patients showed a complete pathological response (pCR) as assessed by ypT0 (or Tis) ypN0 status. The number of patients with pCR and residual disease (RD) across all three subtypes showed significantly different distribution (p-value $=0.04$) where the highest pCR rates (41.7%; 10 out of 24) were observed for TNBC patients.

TILs distribution across molecular subtypes

Mononuclear lymphocytes were scored for each tumor tissue based on H&E staining. The spatial distribution of TILs was classified into four phenotypes based on the proximity of lymphocytes to the tumor cells. Intratumoral: lymphocytes infiltrated within the tumor core and in close proximity to tumor cells, peri-tumoral: lymphocytes close to tumor core, but restricted to surrounding stroma, stromal: lymphocytes restricted to stromal tissue and distant from the tumor core and desert: where lymphocytes were absent in tumor core as well as stromal tissue. Representative images of each phenotype are shown in Fig. 1A. Distribution analysis for spatial phenotypes across molecular subtypes revealed TNBC with a higher proportion of intra-tumoral TILs (23%) with hardly 1% patients with desert TILs phenotype. While ER+ patients reflected a high proportion of desert TILs phenotype (32%) and 3% of tumors with intra-tumoral TILs (Fig. 1B). More than 50% of IDC tumors harboured stromal TILs across all subtypes i.e., 56% ($n=50$) for ER+ patients, 50% for both HER2+ and TNBC patients ($n=29$ and $n=40$; respectively). Peri-tumoral TILs were seen in a higher proportion in HER2+ patients compared to other subtypes (34%, $n=20$) (Fig. 1B).

Stromal TILs were quantified by a certified pathologist since more than 50% of the tumors were presented with stromal infiltration of lymphocytes. Percent Stromal TILs (sTILs) infiltration was evaluated as sTILs score for each tumor tissue, and sTILs scores were then compared across the three molecular subtypes (Fig. 1C). TNBC tumors harbored a wider range of sTILs scores (1–90%) as compared to the other subtypes (Fig. 1D). Mean sTILs score was significantly higher in TNBC (32.2 ± 2.8, $n=81$) compared to ER+ and HER2+ tumors (11.9 ± 1.4, $n=90$ and 21.7 ± 2.4, $n=58$, respectively).

Stromal TILs percentage and association with clinical parameters

The distribution of percent sTILs scores across clinical features of the cohort is presented in Table S1. Average sTILs scores showed even distribution across age, menopausal status, and lymph node status. Tumor

Table 1 Demographic table of the breast cancer cohort, with clinical characteristics across subtypes

No. of patients		All BC	ER+	HER2+	TNBC	*p*-values
		229	90	58	81	
Age (*n* = 227)	**(Mean ± S.D)**	54.4 ± 12.2	56.1 ± 12.5	55.1 ± 11.9	51.8 ± 11.9	0.0635*
	Early (> 50)	86 (37.9%)	29 (32.2%)	21 (36.2%)	36 (45.6%)	0.1941
	Late (≤ 50)	141 (62.1%)	61 (67.8%)	37 (63.8%)	43 (54.4%)	
	NA	2	0	0	2	
Menopausal status (*n* = 192)	**pre**	58 (30.2%)	21 (28.0%)	11 (21.6%)	26 (39.4%)	0.0992
	post	134 (69.8%)	54 (72.0%)	40 (78.4%)	40 (60.6%)	
	NA	37	15	7	15	
Grade (*n* = 229)	**Low (I/II)**	111 (48.5%)	67 (74.4.%)	25 (43.1%)	19 (23.5%)	**< 0.0001**
	High (III)	118 (51.5%)	23 (25.6%)	33 (56.9%)	62 (76.5%)	
	NA	0	0	0	0	
Tumor size (cT) (*n* = 216)	**T1**	66 (30.6%)	35 (40.2%)	13 (24.1%)	18 (24%)	0.2913
	T2	136 (63%)	47 (54.0%)	38 (70.4%)	51 (68%)	
	T3	12 (5.5%)	4 (4.6%)	3 (5.6%)	5 (6.7%)	
	T4	2 (0.9%)	1 (1.1%)	0 (0.0%)	1 (1.3%)	
	NA	13	3	4	6	
LN status (cN) (*n* = 206)	**negative**	65 (31.6%)	31 (36.9%)	14 (28.0%)	20 (27.8%)	0.3904
	positive	141 (68.4%)	53 (63.1%)	36 (72.0%)	52 (72.2%)	
	NA	23	6	8	9	
Clinical_Stage (*n* = 209)	**Early(<IIB)**	84 (40.2%)	41 (47.7%)	15 (30%)	28 (38.4%)	0.1185
	Late(≥IIB)	125 (59.8%)	45 (52.3%)	35 (70%)	45 (61.6%)	
	NA	20	4	8	8	
pT (primary tissue, no NACT) (*n* = 128)	**T0**	6 (4.7%)	4 (8.7%)	0 (0.0%)	2 (4.4%)	0.4153
	T1	30 (23.5%)	14 (30.5%)	5 (13.5%)	11 (24.4%)	
	T2	85 (66.4%)	26 (56.5%)	30 (81.1%)	29 (64.4%)	
	T3	4 (3.1%)	1 (2.2%)	1 (2.7%)	2 (4.4%)	
	T4	3 (2.3%)	1 (2.2%)	1 (2.7%)	1 (2.2%)	
	NA/NACT_Yes	101	44	21	36	
pN (primary tissue, no NACT) (*n* = 128)	**negative**	86 (67.2%)	31 (67.4%)	22 (59.5%)	33 (73.3%)	0.4119
	positive	42 (32.8%)	15 (32.6%)	15 (40.5%)	12 (26.7%)	
	NA/NACT_Yes	101	44	21	36	
pathological Stage (primary tissue, no NACT) (*n* = 128)	**Early(<IIB)**	85 (66.4%)	31 (67.4%)	21 (56.8%)	33 (73.3%)	0.2819
	Late(≥IIB)	43 (33.6%)	15 (32.6%)	16 (43.2%)	12 (26.7%)	
	NA/NACT_Yes	101	44	21	36	
LVI (*n* = 229)	**negative**	184 (80.3%)	67 (74.4%)	47 (81.0%)	70 (86.4%)	0.1426
	positive	45 (19.7%)	23 (25.6%)	11 (19.0%)	11 (13.6%)	
	NA	0	0	0	0	
NACT (*n* = 218)	**No**	142 (65.1%)	52 (62.2%)	40 (70.2%)	50 (65.8%)	
	Yes	76 (34.9%)	33 (38.8%)	17 (29.8%)	26 (34.2%)	
	NA	11	5	1	5	
PCR status after NACT (*n* = 60)	**pCR**	15 (25%)	3 (11.1%)	2 (22.2%)	10 (41.7%)	**0.0414**
	RD	45 (75%)	24 (88.9%)	7 (77.8%)	14 (58.3%)	
	NA	17	7	8	2	
Survival outcomes	**No. followed-up**	219	87	54	78	
	Time in Months Mean (range)	22(0–172)	18(0–84)	24(0–172)	24(0–132)	
	Median months	14	12	14	18	
	# Recurred (local, distant)	18	5	3	10	
	# Death due to disease	6	2	0	4	

A cohort of IDC patients was grouped according to the ER+, HER2+ and TNBC subtypes. Clinical parameters such as age at diagnosis, menopausal status, tumor grade, radiological and pathological tumor size, lymph node positivity, stage, and LVI are listed. The number of patients is listed according to the clinical variables reported at the time of diagnosis. For patients who did not receive NACT/NAHT, pT and pN retrieved from the surgery pathology report are noted. For patients who received NACT/NAHT, pathological response to the therapy is noted based on their ypTypN status. A total number of patients with follow-up, mean time to follow-up and follow-up status are also noted. The distribution of clinical parameters across the ER+, HER2+ and TNBC subtypes, was analyzed using the 2*3 (4*3 and 5*3 in case of clinical and pathological tumor size) χ2 contingency test with GraphPad Prism v.5. Bold font indicates significa t p-values

*For comparing mean age differences among the subtypes, one way ANOVA was performed

LVI lymphovascular invasion, *NACT* Neoadjuvant chemotherapy, *pCR* pathological complete response

grade showed significantly higher sTILs percentages in high-grade tumors (32.1 ± 2.2) vs low-grade tumors (10.4 ± 0.9). Tumors with smaller sizes showed higher sTILs compared to larger tumors, for both clinical - cT as well as pathological - pT size.

sTILs scores were correlated across molecular subtypes and their clinical parameters (Table 2). Mean sTILs score was significantly higher in TNBC across all the clinical parameters compared to ER+ and HER2+ patients except for lymph node involvement and lymph vascular invasion (LVI). For lymph node involvement and LVI, ER+ and HER2+ but not TNBC tumors show increased infiltration in node-positive tumors in contrast to previously published meta-analysis [24] for both clinical and pathological reports.

TILs distribution and its correlation with response to NACT

Out of the 60 patients that received NACT, a significantly higher proportion of TNBC patients showed pCR (42%) compared to ER+ (11%) and HER2+ (22%) patients (Table 1). To determine the role of TILs in NACT response, an association between the spatial phenotype of TILs as well percent stromal infiltration scores were evaluated.

Patients with pathological complete response showed a higher proportion of intra-tumoral and peri-tumoral TILs phenotypes (27 and 20%, respectively) compared to patients with residual disease (11 and 7%, respectively) (Fig. 2A, B). Tumors ($n=7$) with desert TILs phenotype showed RD, where 100% of the patients were left with residual disease (RD) post-NACT (Fig. 2A, B).

Analysis of spatial phenotype across subtypes shows the highest proportion of ER+ tumors harboring stromal and desert phenotype where patients are left with residual tumor (RD) post-NACT (Fig. 2C), while a small proportion of tumors with stromal TILs had pCR for HER2+ subtype (Fig. 2D). For TNBC tumors, pCR was observed for all spatial phenotypes with a higher percentage of response for intra-tumoral and per-tumoral phenotypes (Fig. 2E).

Next, sTILs scores binned into three categories – low (< 10%), moderate (10–40%) and high (> 40%) according to the TILs working group guidelines [22] were analyzed for its association with response to NACT (Fig. 2F). The highest proportion of patients (50%) with pCR had high sTILs compared to patients with RD, where 81.2% of patients had low sTILs scores. A similar trend was seen for TNBC patients, where 60% of patients with high sTILs score showed pCR.

Patients with complete response showed a wider range of percent sTILs scores compared to the ones who had residual disease (RD) post-NACT (Fig. 2G). TNBC patients with pCR showed a similar trend of higher and wider sTILs scores compared to patients with RD (Fig. 2J), while the opposite trend was observed for ER+ and HER2+ patients (Fig. 2H, I).

sTILs scores comparison between pre- and post-NACT

To evaluate if there is an association between treatment response and changes in stromal infiltration through the treatment, sTILs scores were assessed for any alteration from pre-NACT to a post-NACT setting. The change in the scores between pre- and post-NACT settings are plotted according to the treatment response (Fig. 3B). Patients with pCR ($n=4$) showed a significant decrease in sTILs scores from pre-NACT to post-NACT setting (Fig. 3C), while patients with residual disease (RD) showed no significant change (Fig. 3D). When compared across subtypes, no significant correlation was observed for ER+ and HER2+ tumors (Fig. 3E, F), while TNBC tumors showed a significant association of reduced stromal infiltration from pre-treatment to post-treatment setting with pCR but not with RD (Fig. 3G).

TILs distribution and its association with survival outcomes

Spatial TILs phenotypes (stromal, peri-, intra- and desert) were analyzed for their association with survival outcomes, disease-free survival (DFS) and overall survival

(See figure on next page.)

Fig. 1 TILs spatial phenotype and stromal TILs (sTILs) scores across the three subtypes of breast cancer. **A**. Representative images depicting the spatial distribution of TILs in breast cancer. Representative images of four phenotypes of spatial TILs are presented here for a. Intra-tumoral TILs, b. Peri-tumoral TILs, c. Stromal TILs and d. Desert TILs. The yellow area represents the tumor area. Red arrows indicate TILs. Blue lines are the scale bars representing 100 µm. **B**. Stacked bar graph representing percent number of patients across the subtypes for four phenotypes of spatial TILs. The number of patients and percentage is shown as n (%). Distribution of number of patients across the spatial phenotypes according to their subtypes is tested by 3*4 $\chi 2$ (Chi-Square) contingency test. **C**. Representative images depicting stromal TILs distribution. Representative images of sTILs scores are presented here for ER+ (left panel), HER2+ (middle panel) & TNBC (right panel) with sTILs scores mentioned at the left bottom. Blue lines are the scale bars representing 200 µm. **D**. Box plot shows the distribution of sTILs scores across ER+, HER2+ and TNBC subtypes. The horizontal line represents the median. Error bars represent 10th and 90th percentile values. The number of patient samples (n) are shown in the box plot. The distribution of the sTILs scores amongst subtypes was analyzed for statistical significance with the Kruskal Wallis test and individual comparison between two subtypes by Mann-Whitney test, using GraphPad Prism v.5. *represent *p*-value of < 0.05, *** represents p-value < 0.0005

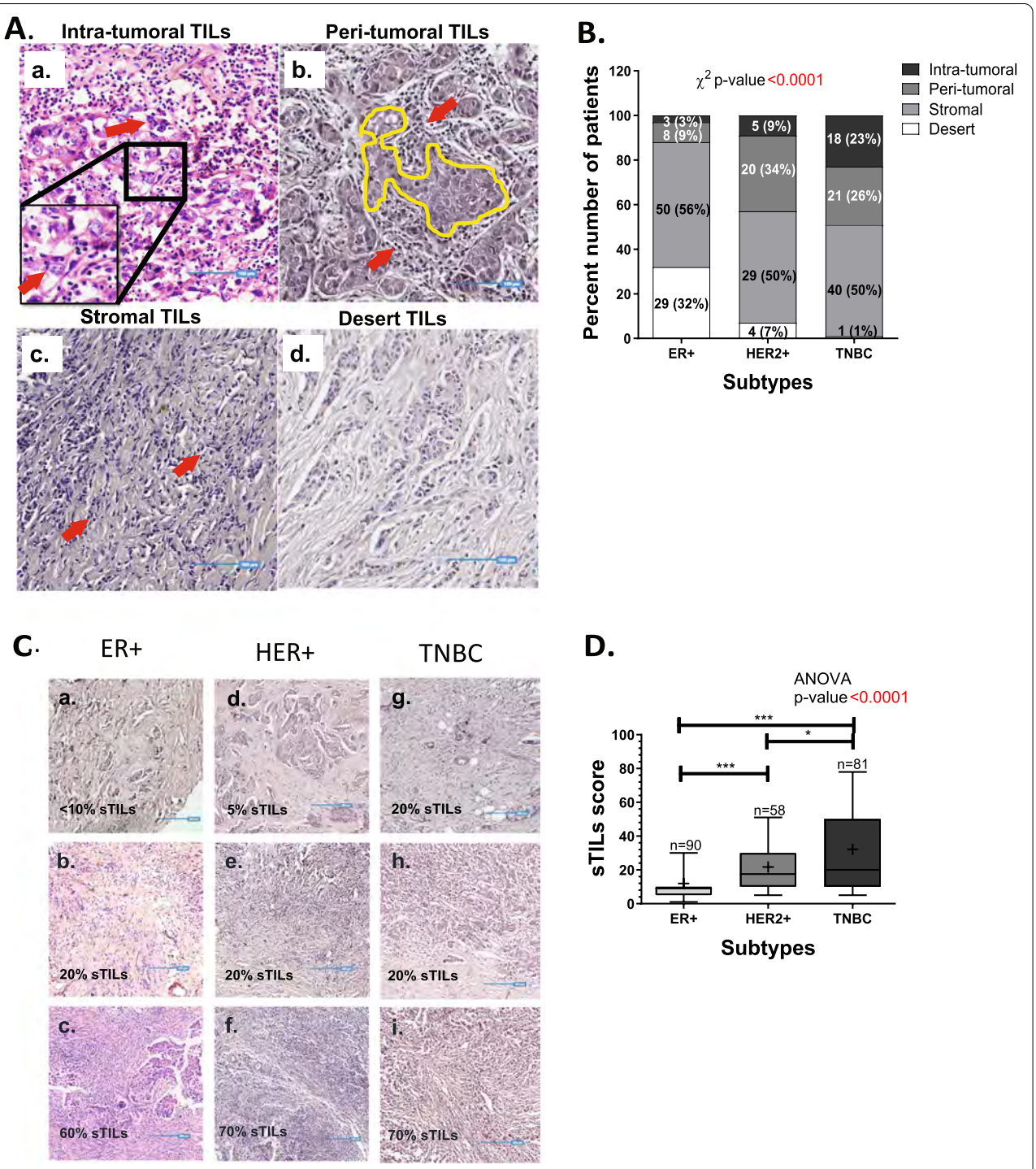


Fig. 1 (See legend on previous page.)

(OS) (Fig. 4 and S3). Surprisingly, for the entire cohort of IDC patients, desert phenotype showed longer event-free survival (DFS), followed by intra-tumoral phenotype, while stromal and peri-tumoral TILs phenotypes showed shorter survival, though not significant (Fig. 4A). ER+ subtype showed worst disease-free survival for patients with stromal TILs phenotype (Fig. 4B), while no specific association was observed for HER2+ subtype. For TNBC patients, this cohort showed better survival for patients with intra-tumoral lymphocyte infiltration over stromal

Table 2 Mean sTILs scores with respect to clinicopathological features of breast cancer subtypes

TILs distribution against subtypes w.r.t clinical parameters		ER+ (*n* = 90)	HER2+ (*n* = 58)	TNBC (*n* = 81)	*p-values (Kruskal-Wallis test)*
No. Of patients (*n* = 229)	**(Mean ± S.E)**	11.9 ± 1.4 (*n* = 90)	21.7 ± 2.4 (*n* = 58)	32.2 ± 2.8 (*n* = 81)	**< 0.0001**
Age, (*n* = 227)	**Early (< 50)**	14.9 ± 3.5 (*n* = 29)	18.1 ± 3.1 (*n* = 21)	34.3 ± 4.1 (*n* = 36)	**< 0.0001**
	Late (≥ 50)	10.5 ± 1.3 (*n* = 61)	23.8 ± 3.4 (*n* = 37)	30.8 ± 3.9 (*n* = 43)	
	NA	0	0	2	
Menopausal status, (*n* = 192)	**Pre**	16.9 ± 4.7 (*n* = 21)	12.7 ± 2.6 (*n* = 11)	31.7 ± 4.9 (*n* = 26)	**< 0.0001**
	Post	10.3 ± 1.5 (*n* = 54)	24.4 ± 3.3 (*n* = 40)	33.8 ± 4.3 (*n* = 40)	
	NA	15	7	15	
Grade, (*n* = 229)	**I/II**	7.9 ± 0.8 (*n* = 67)	12.0 ± 1.8 (*n* = 25)	17.1 ± 3.5 (*n* = 19)	**< 0.0001**
	III	23.7 ± 4.4 (*n* = 23)	29.1 ± 3.6 (*n* = 33)	36.8 ± 3.3 (*n* = 62)	
	NA	0	0	0	
Tumor size (cT), (*n* = 216)	**T1, T2**	12.2 ± 1.5 (*n* = 82)	22.1 ± 2.7 (*n* = 51)	32.7 ± 3.1 (*n* = 69)	**< 0.0001**
	T3, T4	11.8 ± 4.7 (*n* = 5)	18.3 ± 7.3 (*n* = 3)	22.5 ± 6.6 (*n* = 6)	
	NA	6	8	9	
LN status (cN), (*n* = 206)	**Negative**	10.5 ± 2.2 (*n* = 31)	16.4 ± 4.1 (*n* = 14)	34.4 ± 5.9 (*n* = 20)	**< 0.0001**
	Positive	13.3 ± 2.1 (*n* = 53)	25.0 ± 3.4 (*n* = 36)	29.6 ± 3.4 (*n* = 52)	
	NA	6	8	9	
Clinical Stage, (*n* = 209)	**Early (<IIB)**	9.7 ± 1.7 (*n* = 41)	16.3 ± 3.8 (*n* = 15)	37.1 ± 5.3 (*n* = 28)	**< 0.0001**
	Late (≥IIB)	14.4 ± 2.4 (*n* = 45)	25.5 ± 3.5 (*n* = 35)	28.9 ± 3.5 (*n* = 45)	
	NA	4	8	8	
LVI, (*n* = 229)	**Negative**	10.8 ± 1.3 (*n* = 67)	20.9 ± 2.7 (*n* = 47)	32.7 ± 3.0 (*n* = 70)	**< 0.0001**
	Positive	15.3 ± 4.3 (*n* = 23)	25.0 ± 6.1 (*n* = 11)	28.7 ± 8.1 (*n* = 11)	
	NA	0	0	0	
Tumor size (pT), (*n* = 128) NACT_No	**T0, Tis**	23.8 ± 12.5 (*n* = 4)	0	50.0 ± 30.0 (*n* = 2)	**< 0.0001**
	T1, T2	10.2 ± 1.7 (*n* = 40)	19.1 ± 3.0 (*n* = 35)	33.3 ± 4.2 (*n* = 40)	
	T3, T4	17.5 ± 12.5 (*n* = 2)	20.0 ± 10.0 (*n* = 2)	20.0 ± 10.0 (*n* = 3)	
	NA/NACT_Yes	44	21	36	
LN status (pN), (*n* = 128) NACT_No	**Negative**	10.5 ± 2.2 (*n* = 31)	17.7 ± 3.5 (*n* = 22)	34.8 ± 4.7 (*n* = 33)	**< 0.0001**
	Positive	14.1 ± 3.8 (*n* = 15)	21.3 ± 5.0 (*n* = 15)	28.8 ± 7.3 (*n* = 12)	
	NA/NACT_Yes	44	21	36	
Pathological Stage, (*n* = 128) NACT_No	**Early (<IIB)**	10.5 ± 2.2 (*n* = 31)	18.1 ± 3.7 (*n* = 22)	35.4 ± 4.6 (*n* = 33)	**< 0.0001**
	Late (≥IIB)	14.1 ± 3.8 (*n* = 15)	20.6 ± 4.8 (*n* = 16)	27.1 ± 7.4 (*n* = 12)	
	NA/NACT_Yes	44	21	36	

Mean ± S.E sTILs scores according to the molecular subtypes are presented across clinicopathological parameters including age at diagnosis, menopausal status of patients, tumor grade, radiological and pathological tumor size, lymph node status and stage, and LVI

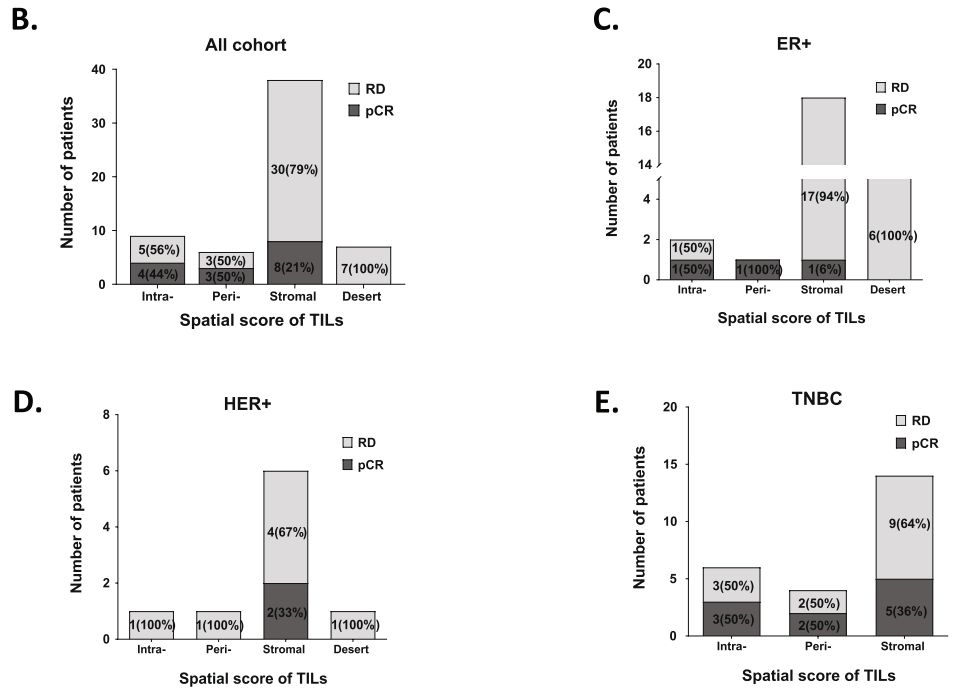
The statistical analysis was performed using GraphPad Prism v.5. Kruskal Wallis test was performed to compute the significan e for mean sTILs scores across breast cancer subtypes. The bold font indicates significa t p-values. *LVI- lymphovascular invasion

(See figure on next page.)

Fig. 2 Spatial TILs phenotype and sTILs scores association with NACT response. **A** Spatial TILs phenotype and its association with response to NACT. Table showing the number of patients according to their TILs spatial phenotype and pathological response, where the response is measured as pCR and RD. Distribution of the number of patients across four phenotypes of spatial TILs was analyzed with the 4*2 χ2 contingency test using GraphPad Prism v.5. The bold font indicates significant *p*-values. **B-E** Spatial TILs phenotypes and its association with response to NACT across subtypes. Stacked bar graph representing percent number of patients with each spatial TILs phenotype with respect to NACT response. The therapy response is reported as pCR and RD according to the spatial TILs phenotype of the tumor for B. the IDC cohort, C. ER+ subtype D. HER2+ subtype E. TNBC subtype. The number of patients and percentage is shown in each bar as n (%). **F** Table showing the distribution of IDC and TNBC patients with pCR or RD with respect to binned sTILs score; Low sTILs (< 10%), Moderate sTILs (10–40%) and High sTILs (≥40%). χ2 p-value was computed using GraphPad Prism V.5. G-J; Box plots depicting mean sTILs scores separated according to the response to NACT for the cohort and the three subtypes. The number of tissue samples (n) is shown on top of each bar. Error bars represent 10th and 90th percentile values. Mann Whitney test was performed to analyze significant distribution of mean sTILs scores. *p*-value < 0.05 is represented with '*', < 0.01 with '**' and, < 0.0001 with '***'. ns = non-significant. GraphPad Prism v.5 was used for the graphs and statistical calculations

A.

All BC (NACT_Yes) (n=76)	pCR (n=15)	RD (n=45)	NA (n=16)	χ2 p-value
Intra-tumoral TILs (n=9)	4 (27%)	5 (11%)	0	0.0980
Peri-tumoral TILs (n=10)	3 (20%)	3 (7%)	4	
Stromal TILs (n=48)	8 (53%)	30 (67%)	10	
Desert TILs (n=9)	0 (0%)	7 (15%)	2	



F.

	All BC	Low sTILs (<10%)	Moderate sTILs (10%-40%)	High sTILs (≥40%)	χ2 p-value
All BC	pCR	3 (18.8%)	8 (22.2%)	4 (50%)	0.2072
	RD	13 (81.2%)	28 (77.8%)	4 (50%)	
TNBC	pCR	2 (40%)	5 (35.7%)	3 (60%)	0.6372
	RD	3 (60%)	9 (64.3%)	2 (40%)	

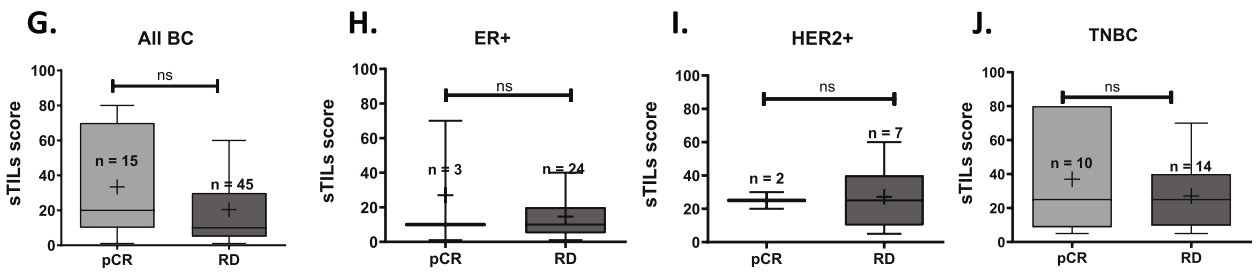


Fig. 2 (See legend on previous page.)

or peri-tumoral (Fig. 4D). For overall survival across the cohort and subtypes, again, no specific association was observed for spatial TILs phenotypes (Fig. S3).

Further, following TILs working group guidelines, percent stromal TILs scores were binned into three categories: low (< 10%), moderate (10–40%) and high

(> 40%) and were analyzed for association with the survival outcomes (Fig. 4 and S3). The cohort showed no specific association of binned sTILs scores with disease-free survival (Fig. 4E). Subtype wise analysis with respect to binned sTILs scores showed high sTILs scores in TNBC tumors to be significantly associated with longer disease-free survival as compared to low and moderate sTILs scores (Fig. 4H). ER+ patients showed a similar trend as that of TNBC, though ER+ subtypes had very few patients with high sTILs scores (Fig. 4F). In contrast, high sTILs showed poor survival in HER2+ patients (Fig. 4G). For overall survival, the cohort comprised of 6 events (death due to disease), where 5 out of 6 harbored moderate sTILs scores, while patients with high sTILs did not have any events within the follow-up period (Fig. S3 D-F).

Discussion

Infiltrating tumor lymphocytes are being evaluated in clinical trials as a surrogate marker for treatment response in breast cancer, as summarized recently by TILs working group [26] and specifically in TNBC [27]. TILs scores serve as a potential prognostic marker, not only as a surrogate marker to de-escalate toxic and expensive chemotherapy, but also due to its cost-effectiveness as a diagnostic tool, especially in low-resource countries such as India. With this anticipation, this study presents one of the first TILs evaluations in an Indian cohort of breast cancer patients. Detailed evaluation of lymphocytes' spatial distribution and stromal infiltration scores for their association with treatment response and patient outcomes is presented here.

The cohort of 229 breast cancer with 81 TNBC, 90 ER+ and 58 HER2+ patients reflected the uniform distribution of clinicopathological parameters except for age and grade. TNBC patients presented with younger mean age as compared to other subtypes and comprised of a higher proportion (45%) of young age (< 50 years) and (39%) premenopausal patients. Younger age at incidence for TNBC subtype in high frequency is reported earlier in two meta-analyses for breast cancer patients in India [7, 9]. The cohort shows a similar and significant trend of age distribution across breast cancer patients. This is in contrast to the western cohorts where young age TNBCs present at 29–34% [28, 29].

TILs in the tumor microenvironment influence the overall breast cancer prognosis and response to treatment [26]. CD8+ T-cell population has been used to define spatial phenotypes in breast cancer based on the infiltration in the tumor core and/or stroma [30]. A higher proportion of cytotoxic T cells in the tumor core has been shown to be associated with a better prognosis. Therefore, the spatial context of TILs with respect to the tumor was analyzed for the Indian cohort for its association with response to NACT and survival outcomes. More than 50% of patients in each subtype presented with stromal TILs, while 11.3% patients harbored intra-tumoral TILs ($n = 26$), where intra-tumoral TILs associated with better outcomes in IDC patients. Specifically in TNBC subtypes, higher proportion of tumors (23%) harbored intra-tumoral TILs, unlike the western cohort, where iTILs were observed in a small proportion of IDC as well as TNBC patients [22]. In BIG 02–98 trial data, median intra-tumoral infiltration: iTILs score of 2%, while median stromal TILs score was 10% for a cohort size of 2000 patients was observed [31]. With a higher number of patients with intra-tumoral TILs, specifically in the TNBC subset of the cohort, their prognostic role needs to be further evaluated within Indian cohorts.

Two comprehensive meta-analyses for stromal TILs association with patient outcomes for large cohorts of breast cancer patients have been studied earlier [23, 24]. Denkert et al. in 2018, where pooled data from 6 clinical trials with 3771 patients where sTILs scores are binned as 60% and above as the cut-off for high sTILs subgroup [23]. In another meta-analysis by Loi et al., sTILs scores for 2148 TNBC patients were analyzed with high sTILs cut-off as 30% and above [24]. Despite different cut-off

(See figure on next page.)

Fig. 3 sTILs scores compared between pre-NACT and post-NACT tumor tissue. **A** Table represents mean ± S.E sTILs scores across clinicopathological parameters, including radiological and pathological tumor size, lymph node status for NACT-treated patients, according to the molecular subtypes. The statistical analysis was done using GraphPad Prism v.5. Kruskal Wallis test was performed to compute the significant difference in mean sTILs scores across breast cancer subtypes. The bold font indicates signifiant *p*-values. **B-G** Before-after graph depicting sTILs scores for pre- and post-NACT tumor tissue. Individual sTILs scores for each paired sample is shown for patients who received NACT. B; the IDC cohort, C; patients with pCR and D; patients with RD, E; the IDC cohort, E; ER+ subtype, F; HER2+ subtype and G, TNBC subtype. Paired t-test was performed to test the difference in mean between sTILs scores of primary and post-NACT tissue. The red lines indicate patients who showed pathological complete response (pCR), and black lines indicate patients who had the residual disease (RD). The bold font indicates a significant *p*-value. *p*-value < 0.05 is represented with '*', < 0.01 with '**' and, < 0.0001 with '***'. ns = non-significant. GraphPad Prism v.5 was used for the graphs and statistical calculations

A.

TILs distribution against subtypes w.r.t clinical parameters		ER (n=33)	HER2 (n=17)	TNBC (n=26)	*p-values (Kruskal-Wallis test)*
No. of patients, (n=76) NACT_Yes	(Mean ± S.E)	13.8 ± 2.8 (n=33)	29.1 ± 5.0 (n=17)	30.5 ± 4.9 (n=26)	**0.0007**
Tumor size (cT), (n=74) NACT_Yes	T1,T2 T3,T4 NA	14.1 ± 3.2 (n=28) 11.8 ± 4.7 (n=5) 0	30.3 ± 5.5 (n=15) 30.0 ± 0.0 (n=1) 1	31.8 ± 5.6 (n=22) 25.0 ± 10.4 (n=3) 1	**0.0121**
LN status(cN), (n=73) NACT_Yes	negative positive NA	5.0 ± 1.9 (n=4) 15.0 ± 3.1 (n=29) 0	0 31.3 ± 5.3 (n=15) 2	0 30.9 ± 5.0 (n=25) 1	**0.0006**
Clinical Stage, (n=74) NACT_Yes	Early (<IIB) Late (≥IIB) NA	15.6 ± 9.2 (n=8) 15.5 ± 3.3 (n=27) 0	10.0 ± 0.0 (n=1) 34.6 ± 5.6 (n=13) 3	80.0 ± 0.0 (n=1) 28.9 ± 4.8 (n=24) 1	**0.0054**
Tumor size (ypT), (n=60) NACT_Yes	T0, Tis T1,T2 T3,T4 NA	28.2 ± 13.5 (n=5) 14.3 ± 3.5 (n=16) 10.0 ± 3.5 (n=4) 8	25.0 ± 5.0 (n=2) 27.1 ± 7.1 (n=7) 10.0 ± 0.0 (n=1) 7	39.1 ± 9.3 (n=11) 24.2 ± 5.0 (n=14) 0 1	0.2281
LN status(ypN), (n=61) NACT_Yes	negative positive NA	18.1 ± 6.6 (n=10) 14.4 ± 3.8 (n=16) 7	30.0 ± 7.5 (n=5) 16.0 ± 4.9 (n=5) 7	32.4 ± 6.2 (n=19) 25.7 ± 8.1 (n=6) 1	0.082
Pathological Stage, (n=60) NACT_Yes	Early (<IIB) Late (≥IIB) NA	16.3 ± 5.6 (n=12) 16.2 ± 4.5 (n=13) 8	26.3 ± 6.2 (n=8) 20.0 ± 10.0 (n=2) 7	30.6 ± 5.5 (n=22) 31.7 ± 15.9 (n=3) 1	0.2559

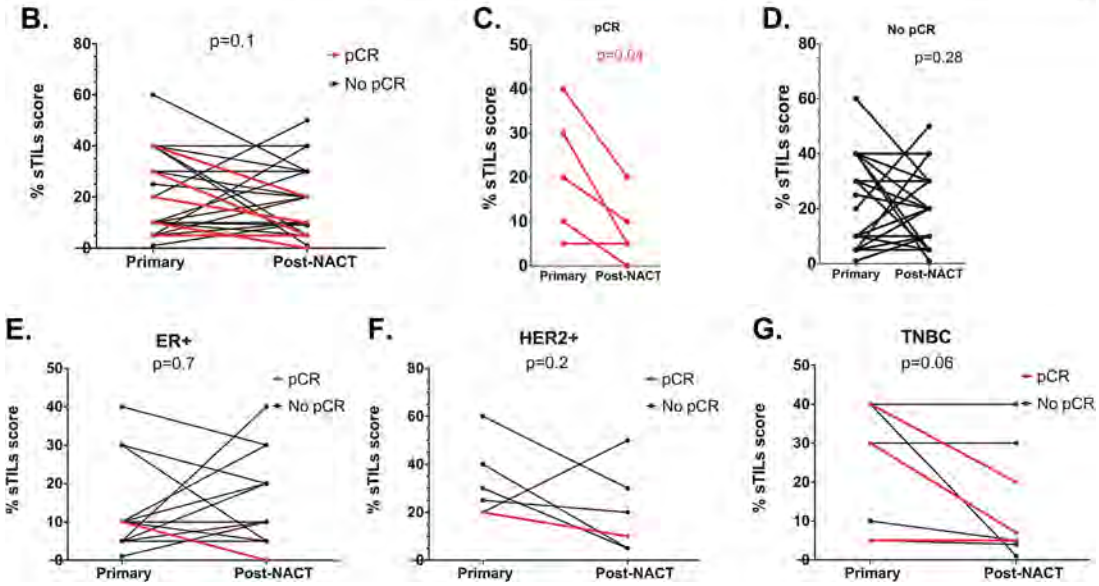


Fig. 3 (See legend on previous page.)

for high sTILs at 40%, subtype wise comparison of sTILs scores from our cohort co-related well with reported studies [22, 23], where TNBC subtype presented with higher mean sTILs scores compared to that of ER+ and HER2+ subtypes.

Stromal TILs scores in the cohort were uniformly distributed irrespective of the clinic-pathological parameters of the tumors, except for grade and tumor size. Higher sTILs were seen in grade 3 TNBC tumors. Similar association was seen by Loi et al., where a higher sTILs score was significantly associated with high-grade tumors [24]. Within TNBC, higher sTILs scores co-related with better disease-free outcomes, as reported earlier by Denkert and colleagues [23].

For patients who received NACT ($n=60$), 42% of TNBC patients showed complete pathological response post-NACT as opposed to 11 and 22% of ER+ and HER2+ subtypes, respectively. This is in line with the literature, where the TNBC subtype has been reported to have a better response to therapy [3, 13, 32]. Even with the limited number of patients that received NACT, higher sTILs scores were associated with complete pathological response. Partial or lack of response to therapy co-related with a lower sTILs scores specifically for the TNBC subtype, similar to that has been reported in western population [23]. The trends observed in our cohort analysis are in line with the established association of sTILs distribution and TNBC outcomes.

In this study, changes in stromal infiltration of lymphocytes in tumor tissue from pre- to post-treatment settings were assessed between paired samples. Interestingly, a significant decrease in the lymphocyte infiltration was observed for patients who showed complete pathological response post-NACT. There is another study where a cohort of 104 TNBC patients [33] showed that increase in sTILs in post-NACT samples associated with better disease-free survival compared to patients where a decrease in sTILs was observed. With such contrasting observations within small sets of cohorts, whether such changes in sTILs in a post-NACT setting can be directly implicated in complete response and better outcomes needs to be evaluated further.

This is the first time an Indian cohort of breast cancer patients is evaluated to assess whether the predictive benefit of TILs can be extended towards breast cancer patients in India, especially when Indian cohorts show significantly different demographic distribution. Our analysis reflects similar trends for TILs association with clinical parameters and patient outcomes despite the demographic differences. Though, finer differences are revealed in this analysis, such as a higher proportion of patients with intra-tumoral TILs with better outcomes over stromal TILs. Thus, extending the utility of the TILs as a putative predictive marker for treatment response for TNBC in India will require further validation with a larger cohort.

TILs assessment can be done using inexpensive and traditional histopathology methods, routinely used even in low-resource countries like India. TILs assessment from histopathology images of tumor slides can be developed as a robust digital pathology tool that can be incorporated for treatment utility predictions and can be availed across the country.

With further validation in a larger cohort across India, TILs have the potential to be a predictive biomarker for chemotherapy response. The newer treatments, like immune checkpoint inhibitor therapies that depend on PD1/PDL1 diagnostic markers, have been shown to use TILs as an associative marker for PD1/PDL1 expression in recent trials such as KEYNOTE-086 [34] and IMPASSION130 [35]. PD1/PDL1 diagnosis is expensive in India and hence with very low penetrance of utility due to low-recourse settings and a high proportion of low-income communities. If TILs is approved to be a surrogate marker for PD1/PDL1 expression, it will serve as a cost-effective tool for treatment management decisions.

(See figure on next page.)

Fig. 4 Disease-free survival (DFS) for five-year follow-up according to the spatial TILs phenotype and sTILs scores. Disease-free survival (DFS) was calculated as number of months from the date of surgery till the recurrence diagnosis date or last follow-up date up to five years. Kaplan-Meier survival plots for disease-free survival (DFS) are plotted. Each drop shown as a vertical line represents an event i.e., local, or distant recurrence. Survival probability with respect to the spatial TILs phenotype is analyzed using IBM SPSS Statistics v. 21.0.0.0. The number of patients at risk at each time interval of 10 months from 0 to 60 months is shown. The number of events is indicated in brackets at respective time points. **A-D** DFS for the four phenotypes of the spatial TILs; Intra-tumoral TILs, Peri-tumoral TILs, Stromal TILs, and Desert TILs for A; the IDC cohort, B; ER+ subtype C; HER2+ subtype and D; TNBC subtype. In the graph, X-axis represents the time scale in months, and Y-axis represents the survival probability. The green line indicates patients with stromal TILs, the blue line indicates patients with peri-tumoral TILs, the purple line indicates patients with intra-tumoral TILs, and the red line indicates patients with desert TILs phenotype. **E-H** DFS with respect to binned percent stromal TILs infiltration score. Kaplan-Meier survival plots for disease-free survival (DFS) according to low, moderate & high sTILs score bins for E; the IDC cohort, F; ER+ subtype, G; HER2+ subtype and H; TNBC. In the graph, X-axis represents the time scale in months, and Y-axis represents the survival probability. The blue line indicates patients with low sTILs scores, and the red line indicates patients with moderate scores & green indicates high sTILs scores

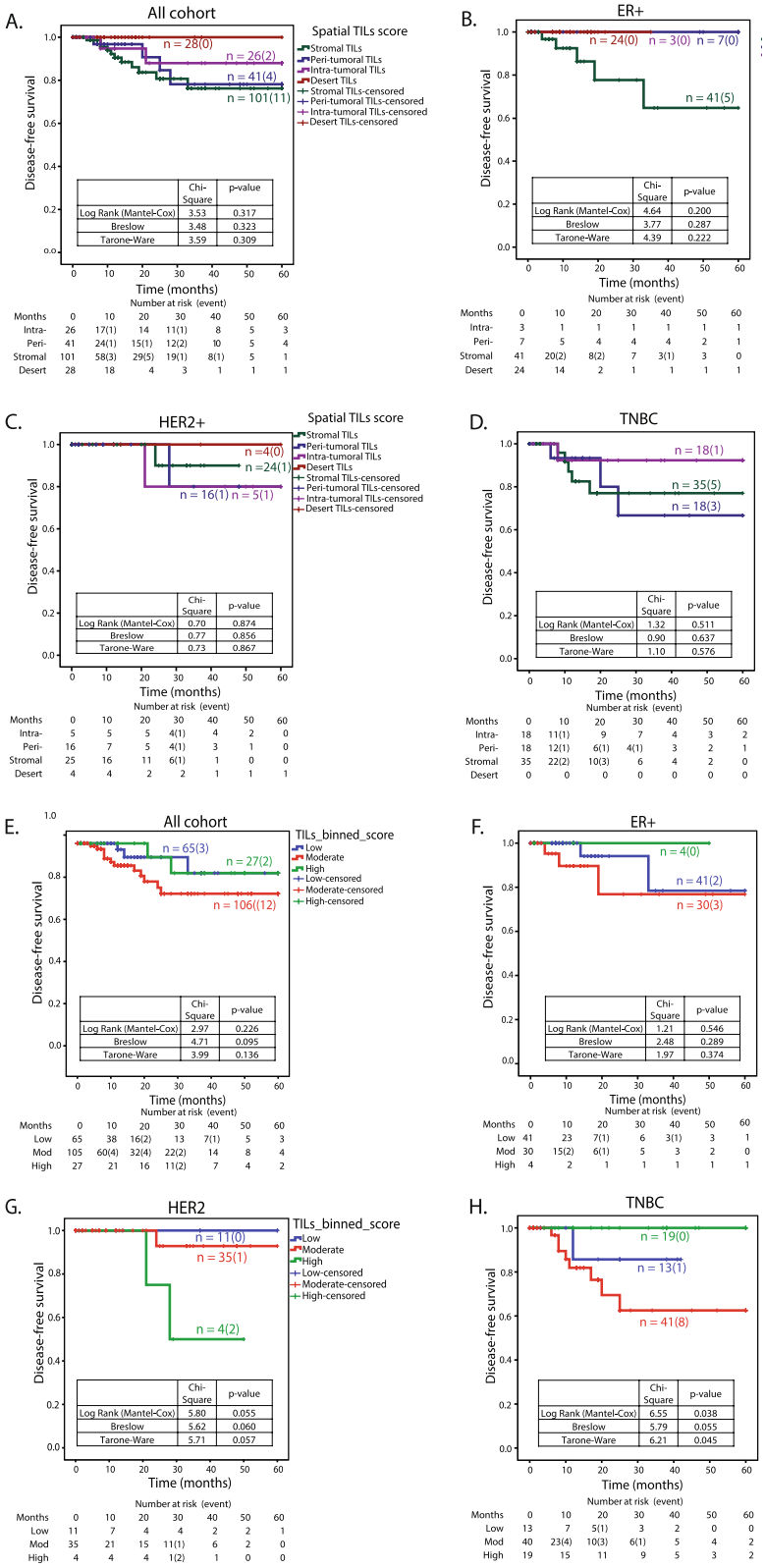


Fig. 4 (See legend on previous page.)

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13000-022-01271-y.

Additional file 1.

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Conflict of interest
The authors declare no conflict of interest.

Authors' contributions
PV was involved in the actual experimentation and study methodology, coordination with the clinical team for clinical data, formal data analysis, and validation and writing of the manuscript. AP and NJ were involved as pathologists and scored the tissue slides. RB, DA, RR, RU, and NN were involved with patient data curation, and DK supervised clinical data curation and was involved in data analysis. PV, MK and DK were involved in the interpretation of the results. LSS, CBK and MK supervised the study and provided the funding and the resource. MK conceptualised the study, supervised the study's investigation and analysis, and reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript.

Declarations

Competing interests
The authors declare that they have no competing interests.

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