Tumor infiltrating lymphocytes in triple negative breast cancer receiving neoadjuvant chemotherapy

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AIM
To determine influence of neoadjuvant-chemotherapy (NAC) over tumor-infiltrating-lymphocytes (TIL) in...
Tumor infiltrating lymphocytes (TILs) have been extensively studied in triple-negative breast cancer (TNBC). Despite the international TILs working group defined harmonization criteria to evaluate TILs, there is some areas that still require a better understanding. One of these areas is the value of TILs in small pieces of tumor like tissue microarrays (TMA), as well as the value and the appropriate methodology to evaluate TIL subpopulations in TMA.

Finally, some recent studies also suggest a prognostic role of TIL in post-NAC samples, however, there is need for more information in this area because evaluation of these samples has special challenges to pathologists as neoadjuvant chemotherapy produces a spectrum of histopathologic changes including decrease in cancer cell number and changes in stroma composition that includes fibrosis, elastosis, collagenization, hyalinization, microcalcification, neovascularization, fibrinoid necrosis and mucinous changes.

This study aims to evaluate TIL variation during NAC through H and E in full-face and in TMA sections as well as the variation of TIL subpopulations through immunohistochemistry (IHC) staining in TMA sections.
MATERIALS AND METHODS

Patients and sample selection
We retrospectively reviewed the files of all new BC cases which came to the Instituto Nacional de Enfermedades Neoplasicas between 2005 and 2010, and we selected 98 TNBC cases with Clinical Stage II - III who went to surgery of breast tumor and axilla after receiving NAC. All core biopsy prior to NAC and breast tumor excision specimens were fixed in 10% neutral buffered formalin and embedded in paraffin at Instituto Pathology Department Archive. Institutional review board approved the protocol of this study.

Staining and quantification of H and E, CD3, CD4, CD8 and forkhead box protein 3 IHC staining
Tumor areas were selected and a 0.6 cm punch from the formalin-fixed paraffin-embedded specimens were obtained and organized in 8-10 cylinder cards (TMA). H and E staining was performed in the full-face and in the TMA sections, and TIL was evaluated as the percentage of the stroma area of tumor that contained lymphocytic infiltrate through a 10% increment system under 200 × - 400 × magnification.

Process of IHC preparation included cutting 4 μm sections from the TMA, deparaffinizing, rehydrating and processing sections using an automatized stainer (Autostainer Link 48, DAKO, Carpinteria, CA, United States) through standard methods. The following antibodies: CD3 (IS503, Dako), CD4 (IS649, Dako), CD8 (IS623, Dako) and FOXP3 (clone: 236A/E7) were used for staining of TMA section. Lymphocyte subsets were calculated through the percentage between lymphocytes/ tumor cells in a 10% increment system, and through the absolute count of the lymphocytes in 5 high power fields under 200 × - 400 × magnification.

Clinical information and pathological response
We obtained clinical information from patient files archived at Instituto Nacional de Enfermedades Neoplasicas. The pathological therapeutic response of the surgically resected tumor was evaluated after NAC. The surgical specimens of breast lesions were cut into 5 mm slices and processed with H and E staining. A PCR was defined as the absence of all invasive cancer cells in breast and axillary lymph nodes, regardless of the presence of non-invasive cancer cells. The mean percentage as well as the mean absolute count of the lymphocytes in 5 high power fields under 200 × - 400 × magnification.

Statistical analysis
The mean percentage as well as the mean absolute number of immune cells was calculated, and those lesions above this mean were graded as increased. Analysis were also performed considering a high percentage of immune cells when were at least 50%. All statistical analyses were performed using STATA software version 12. Associations among variables were evaluated using Fisher’s exact test or the χ² test. The Mann–Whitney U and Spearman’s correlation tests were used to compare groups. The measurement of agreements between TIL evaluated in whole slide and TMA was used intraclass correlation coefficient (ICC). Kaplan-Meier estimation curves disease free and overall survival was applied. All tests were two sided, and a P ≤ 0.05 was considered statistically significant.

RESULTS

Assessment of TILs by H and E
The characteristics of 91 pre-NAC and 80 post-NAC TN BC cases are reported in Table 1. Most cases were ductal infiltrating carcinoma (96.9%), inflammatory (29.6%), clinical stage III (86.7%) and HGIII (75.5%). Most cases received neoadjuvant doxorubicin and paclitaxel (87.7%) and 29.6% obtained pCR. Sixty-six percent of the patients underwent mastectomy. After a median follow-up of 37.5 mo, there were 42% recurrences and 45% deaths. Pathologic complete response was associated to OS (P = 0.0071) but was not associated to DFS (P = 0.1050).

Median pre-NAC TIL percentage in the full-face (n = 91) and in the TMA section (n = 30) was 40 ± 20 and 20 ± 15, respectively. Median post-NAC TIL percentage in the full-face (n = 80) and in the TMA section (n = 58) was 20 ± 15 and 10 ± 5, respectively (Table 2).

Pre-NAC TIL evaluated in full-face had low grade of agreement with TMA sections (n = 30) (ICC = 0.017). Post-NAC samples were larger and allowed to be divided in homogeneous (n = 26) and heterogeneous lesions (n = 26). Heterogeneous lesions had low level of agreement between full-face and TMA sections (ICC = 0.20), and homogeneous lesions had high level of agreement between full-face and TMA sections (ICC = 0.73).

Higher median pre-NAC TIL (n = 91) evaluated in full-face sections was associated to pCR (40% vs 30%, P = 0.0251), DFS (40% vs 20%, P = 0.0076) and OS (40% vs 30%, P = 0.0334); but not to age (P = 0.1427) nor inflammatory features (P = 0.6401), in the univariate analysis. Association between median TIL and pCR remained significant even with adjustment for age. Higher median pre-NAC TIL (n = 30) evaluated in TMA section was only associated to absence of inflammatory features (10% vs 30%, P = 0.0387).

Median post-NAC TIL evaluated in full-face (n = 80) or in TMA section (n = 58) was not associated to any of the previously mentioned features. Post-NAC median H and E TIL percentage evaluated in full-face section was similar in residual fibrous lesions (n = 17) (pCR) and in residual tumor lesions (n = 63) (no pCR) (20% vs 20%, P = 0.6331) (Table 3).

Classification of TIL with a cut-off of 50% did not identify a population associated to any of the previously mentioned features (P = 0.16 for pCR, P = 0.14 for DFS and P = 0.64 for OS).

NAC produced a statistically significant decrease in median TIL percentage when evaluated in full-face section (n = 73 P < 0.0002), but not when evaluated in TMA sections (n = 16 P = 0.4321) (Table 4).
Assessment of TIL subsets by IHC

Analysis of TIL subsets through IHC was calculated through percentage calculation and absolute counting methodology in TMA sections. Percentage calculation was significantly correlated with absolute counting for all markers in pre-NAC (CD3 n = 27 r = 0.7182, CD8 n = 27 r = 0.6064, FOXP3 n = 26 r = 0.7192) and in post-NAC (CD3 n = 55 r = 0.7733, CD4 n = 30 r = 0.6129, CD8 n = 55 r = 0.7338, FOXP3 n = 47 r = 0.5387) TMA sections that had enough material for both quantification methodologies (Table 5). The lymphocyte subset with highest absolute counts in the pre-NAC and post-NAC samples was CD8 (127 ± 193.5 and 156.5 ± 90.5) (Table 4).

Higher absolute counts of CD3, CD4, CD8 and FOXP3 in pre-NAC samples were associated with longer DFS (n = 28 P = 0.003, n = 19 P = 0.0062, n = 28 P = 0.0096 and n = 29 P = 0.0019; respectively). Higher absolute counts of CD3 in pre-NAC samples had longer OS (n = 28 P = 0.0241).

Higher absolute counts of CD4 in post-NAC samples was associated with age (n = 54 P = 0.0393) and pCR (n = 54 P = 0.0095).

Higher ratio of absolute counts of CD8/CD4 in pre-NAC and post-NAC samples was associated with pCR (n = 17 P = 0.0343 and n = 43 P = 0.0086 respectively).

Higher ratio of absolute counts of CD4/FOXP3 in pre-NAC sample was associated with longer DFS (n = 16 P = 0.0389). Higher ratio of absolute counts of CD4/FOXP3 in post-NAC sample was associated with pCR (n = 30 P = 0.003).

Higher ratio of absolute counts of CD4/CD3 in post-NAC samples was associated with pCR (n = 48 P = 0.0095).

DISCUSSION

We have evaluated pre and post-NAC samples in an effort to produce a comprehensive analysis of the role of TIL variation during NAC in TNBC samples. Evaluation of pre-NAC H and E staining of full-face sections found that those tumors with higher TILs are associated to both pCR and better outcome. These results are similar to those found by Denkert et al in the neoadjuvant setting and by Loi et al and Adams et al in the adjuvant setting, and confirm accuracy of our methodology.

We did not find an association between prognosis and TILs in post-NAC samples evaluated in full-face or in TMA sections. García-Martínez et al evaluated 121 BC cases and found that high TIL level in pre-NAC samples was associated to pCR. TIL in pre-NAC and post-NAC were not associated to outcome. By other side, Dieci et al evaluated 278 TNBC with residual disease after NAC and found that those residual lesions with high level of TIL had better prognosis.

We found that TIL percentage evaluated in full-sections were higher in pre-NAC than post-NAC samples. No association between TIL variation (pre- vs post-NAC) and response to NAC was found. Post-NAC samples of those cases who obtained pCR were similar to TIL levels in those cases who did not obtain pCR (residual cancer). By other side, Dieci evaluated 19 selected cases with...
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Table 3  Comparison between tumor-infiltrating-lymphocytes evaluated in full-face and tissue microarrays sections

| Features                  | H and E in the full-face section | H and E in TMA section |
|---------------------------|----------------------------------|------------------------|
|                           | n      | Me ± IQD | P-value | n      | Me ± IQD | P-value |
| Pre-NAC                   |        |          |         |        |          |         |
| Age                       | 91     | 30       | 0.1427  | 30     | 0.6313  |
| ≤ 49                      | 50     | 40 ± 20  |          | 19     | 20 ± 15  |
| > 49                      | 41     | 20 ± 15  |          | 11     | 20 ± 25  |
| pCR                       |        |          | 0.0251  |        |          | 0.2227  |
| No                        | 63     | 30 ± 20  |          | 15     | 30 ± 25  |
| Yes                       | 28     | 40 ± 17.5|          | 15     | 20 ± 10  |
| Inflammatory              |        |          | 0.6401  |        |          | 0.0387  |
| Age                       | 27     | 40 ± 15  |          | 8      | 10 ± 6.5 |
| ≤ 49                      | 64     | 35 ± 20  |          | 22     | 30 ± 15  |
| > 49                      | 46     | 20 ± 20  |          | 20     | 15 ± 13.8|
| pCR                       |        |          | 0.0076  |        |          | 0.1601  |
| No                        | 45     | 40 ± 20  |          | 10     | 25 ± 25  |
| DFS                       |        |          | 0.0334  |        |          | 0.7214  |
| < 32 mo                   | 51     | 30 ± 20  |          | 22     | 20 ± 15  |
| ≥ 32 mo                   | 40     | 40 ± 20  |          | 8      | 20 ± 15  |
| OS                        |        |          | 0.8547  |        |          | 0.5684  |
| < 41 mo                   | 43     | 20 ± 15  |          | 31     | 10 ± 15  |
| ≥ 41 mo                   | 37     | 20 ± 15  |          | 27     | 10 ± 5   |
| DFS                       |        |          | 0.4582  |        |          | 0.1299  |
| Age                       | 54     | 15 ± 10  |          | 16     | 10 ± 0   |
| ≤ 49                      | 63     | 20 ± 15  |          | 44     | 10 ± 7.5 |
| > 49                      | 17     | 20 ± 10  |          | 14     | 10 ± 7.5 |
| pCR                       |        |          | 0.6331  |        |          | 0.1299  |
| No                        | 35     | 20 ± 15  |          | 22     | 10 ± 0   |
| DFS                       |        |          | 0.2450  |        |          | 0.2573  |
| < 32 mo                   | 28     | 20 ± 15  |          | 22     | 10 ± 15  |
| ≥ 32 mo                   | 40     | 20 ± 15  |          | 8      | 20 ± 15  |
| OS                        |        |          | 0.5973  |        |          | 0.6948  |
| < 41 mo                   | 23     | 20 ± 15  |          | 27     | 10 ± 10  |
| ≥ 41 mo                   | 20     | 20 ± 15  |          | 17     | 10 ± 5   |

1 P < 0.05; 2 U Mann Whitney test; 3 Only cases with incomplete pathological response in post NAC. DFS: Disease free survival; Me ± IQD: Median ± interquartile deviation; OS: Overall survival; pCR: Pathologic complete response; NAC: Neoadjuvant-chemotherapy.

Table 4  Comparison of tumor-infiltrating-lymphocyte evaluated in pre- vs post-neoadjuvant-chemotherapy samples

| Population of lymphocytes | n  | Pre-NAC Me ± IQR | Post-NAC Me ± IQR | P-value | P corrected value |
|----------------------------|----|------------------|-------------------|---------|-------------------|
| Percentage                 |    |                  |                   |         |                   |
| H and E whole slide        | 73 | 40 ± 15          | 20 ± 15           | < 0.001 | < 0.002           |
| Absolute counting          |    |                  |                   |         |                   |
| CD3                        | 22 | 244.5 ± 315.5    | 255.5 ± 267       | 0.8583  |                   |
| CD4                        | 7  | 14 ± 94          | 32 ± 43           | 0.6721  |                   |
| CD8                        | 21 | 127 ± 193.5      | 156 ± 90.5        | 0.7544  |                   |
| FOXP3                      | 21 | 18 ± 31          | 12 ± 19.3         | 0.0917  |                   |

1 P < 0.05; 2 Wilcoxon signed rank test; 3 Bonferroni correction. NAC: Neoadjuvant-chemotherapy; TMA: Tissue microarrays; Me ± IQR: Median ± interquartile range.

high TIL level in post-NAC samples and found that lower TIL increased during NAC\(^{[15]}\), and Demaria et al\(^{[16]}\) found that those cases with higher response have an increase of TIL during NAC in a series of 25 BC.

Although lymphocytic infiltration has demonstrated to behave as a prognostic and predictive marker in breast cancer, there are some aspects without standardization. We evaluated TIL in the 0.6 cm TMA, and we found that the TIL percentages differ from those found in the full-face sections. TIL percentage in TMA was not associated to pCR nor prognosis. Breast tumors and especially TNBC are heterogeneous lesions and our findings indicate that TILs concentration is also heterogeneous inside the different tumor areas. The evaluation of only one region of the tumor through a TMA cylinder appears not to produce confident information about immune reaction against the whole tumor.

Different articles have evaluated the role of TIL...
subpopulations (IHC staining), however some of them have measured them by percentage (resembling methodology used for TIL evaluation with H and E)\(^2,3\) and other have measured by an absolute counting\(^1\).

We compared both methodologies in 0.6 cm TMA tumor samples for CD3, CD4, CD8 and FOXP3 lymphocyte subpopulations and we found a significant correlation between both methodologies.

We also evaluated the association between levels of CD3, CD4, CD8 and FOXP3 lymphocyte subpopulations (absolute counting) and clinical features. Although our sample size for evaluating lymphocyte subpopulations is small, we found that higher absolute counts of CD4 lymphocytes in post-NAC samples were associated with pCR. Higher absolute counts of CD3, CD4, CD8 and FOXP3 lymphocytes in pre-NAC samples were associated with DFS, and higher absolute counts of CD3 lymphocytes in pre-NAC samples had longer OS.

Finally, we also found an association of pCR with higher ratio of absolute counts of all CD8/CD4 in pre-NAC, and CD8/CD4, CD4/CD3, CD4/FOXP3 in post-NAC samples. Higher ratio of absolute counts of CD4/FOXP3 in pre-NAC sample was associated with longer DFS.

The role of one TIL subpopulation and the role of the relationship between two TIL subpopulations over tumor behavior have been previously described and some of authors confirm our findings. Rathore et al\(^9\) found that higher levels of CD3\(^+\), CD4\(^+\) and CD8\(^+\) TILs was significantly associated with good prognosis in a series of 123 early BC cases. Kim et al\(^22\) reported that lower number of CD8\(^+\) TILs in breast tumors were significantly associated with lymph node metastasis, higher stage and high proliferative index in a series of 72 early BC cases. Increased number of FOXP3 lymphocytes was associated to lymph node metastasis, high proliferative index and shorter DFS. Ladoire et al\(^10\) evaluated 56 BC cases who went to NAC and found that high CD8 and absence of FOXP3 infiltration was associated to pCR. Miyashita et al\(^11\) evaluated 131 TNBC patients treated with NAC and found that high CD8 TIL levels and high CD8/FOXP3 ratio in residual tumor had better outcome. García-Martínez et al\(^14\) described a decrease of CD4, an increase of CD8 and an absence of changes of FOXP3 during NAC. High levels of CD3 and CD4 in pre-NAC were associated to pcr. A decrease of CD3 and CD4 during chemotherapy was associated to pCR. A decrease of CD3 during NAC was also associated to better DFS and OS. They also found that higher ratio of CD4/CD8 in pre-NAC was associated to a pCR. They also evaluated six public genominc datasets with around 1000 BC patients treated with NAC and found that higher CD4 count in post-NAC samples was associated to pCR. Finally, they found that high levels of CD3 in post NAC was associated to better DFS\(^2,3\). Other authors have also evaluated the role of TIL ratios, such as CD8/CD4\(^22\) or FOXP3/CD3\(^2,3\), as an alternative approach to better integrate the information provided by each TIL subpopulation\(^14\).

Differences among mentioned authors and our findings could be explained by our small population size, analysis of a not representative area in the TMA samples or changes in CD4\(^+\) TILs phenotype from effectors to suppressors. Therefore, our results need to be validated in larger series. Remarkably, our work is the first to our knowledge to evaluate TIL in BC tumors from Latinoamerican women. And, it is the first to compare the evaluation of TIL percentage in full-sections and in TMA sections, as well as to compare the evaluation of TIL levels through percentage analysis and through absolute counting.

Identification of biomarkers and evaluation of therapy in the neoadjuvant setting has become a major challenge in BC since they could speed-up the development and approval of new drugs\(^24\). pCR is a validated surrogate for drug efficacy in the neoadjuvant setting but its specificity needs still to be improved. The finding of a biomarker related to host immunity in the pre or post-NAC samples could have the benefit to predict response not only to chemotherapy but also to immune checkpoint modulators.

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**COMMENTS**

**Background**

Triple-negative-breast-cancer (TNBC) is associated to poor outcome and is highly prevalent among Latinoamerican women. Tumor-infiltrating-lymphocytes (TILs) have been associated to higher response to chemotherapy and better outcome in TNBC when evaluated in retrospective and prospective series as well as meta-analysis. An international TILs working group defined harmonization criteria to evaluate them in 2015, however, there is still some areas requiring a better understanding. There is not information describing TILs in small pieces of tumor, and the value and the appropriate methodology to evaluate TIL subpopulations in tissue microarrays (TMA). There is small information describing variation of TIL during chemotherapy.

**Table 5 Relationship between percentage and absolute counting methodologies of tumor-infiltrating-lymphocyte subpopulations**

| Population of lymphocytes | \(n\) | \(\rho^2\) | \(P\)-value | \(P\) corrected value |
|---------------------------|------|---------|------------|----------------------|
| Pre-NAC                   |      |         |            |                      |
| CD3                       | 27   | 0.7182  | < 0.001    | < 0.004\(^1\)       |
| CD4                       | 17   | 0.5071  | 0.0378     | 0.1512               |
| CD8                       | 27   | 0.6064  | 0.0008     | 0.0003\(^2\)        |
| FOXP3                     | 26   | 0.7192  | < 0.001    | < 0.004\(^1\)       |
| Post-NAC                  |      |         |            |                      |
| CD3                       | 55   | 0.7733  | < 0.001    | < 0.004\(^1\)       |
| CD4                       | 30   | 0.6219  | 0.0003     | 0.0012\(^2\)        |
| CD8                       | 55   | 0.7338  | < 0.001    | < 0.004\(^1\)       |
| FOXP3                     | 47   | 0.5387  | 0.0001     | 0.0004\(^1\)        |

\(^1\)\(P < 0.05\); \(^2\)Spearman correlation coefficient test; \(^3\)Bonferroni correction.

NAC: Neoadjuvant-chemotherapy.
Research frontiers
TNBC has higher prevalence in Latinoamerican women and is a poor prognostic malignancy without target therapy. Chemotherapy is the only available treatment for TNBC. TIL appears to identify prognosis in TNBC and some recent studies are evaluating if it predicts response to chemotherapy or immunotherapy. Therefore, information about TIL variation during chemotherapy is an important issue as it is the scenario we need to improve treatment efficacy.

Innovations and breakthroughs
As TNBC malignancy and role of TIL as biomarker are important issues, more research about relevance of TIL in TNBC is needed. Therefore, the authors revealed that TIL evaluated in a small area of tumor differs to those evaluated in full-face samples, and lose their prognostic and predictive value. The authors also found that evaluation of lymphocyte subsets can be equally performed through absolute counting or percentage calculating, and can provide prognostic information.

Applications
Based on the present study, the authors can suggest that full-face samples (and not core samples) are used for TIL evaluation in H and E. Absolute counting and percentage calculating could be considered appropriate for evaluation of TIL subsets.

Terminology
TIL is a biomarker with current strong evaluation in different malignancies including TNBC. TIL is accepted as those stromal mononuclear cells inside the tumor but not in contact with cancer cells or inside tumor niches. TMA is a technique allowing immunohistochemistry staining of many tumor samples at the same time.

Peer-review
The paper is very interesting and well written.

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