Immune Analysis of Lymph Nodes in Relation to the Presence or Absence of TILs in TNBC

Ángela Quintana[1,2], Vicente Peg[2,3,4], Teresa Moliné[3], Aleix Prat[5,6], Laia Paré[5,6], Patricia Galván[5,6], Guillermo Villacampa[7], Rodrigo Dientsmann[7], Eva Muñoz-Couselo[1,8], José Perez[9], Merce Marti[2], Juan Blanco-Heredia[10], Carla dos Anjos[10], Miguel Vazquez[11], Leticia De Mattos-Arruda[10], Javier Cortés[1,9,12]

[1]Vall d'Hebrón Institute of Oncology, Barcelona, Spain; [2]Universidad Autónoma de Barcelona, Barcelona, Spain; [3]Department of Pathology, Vall d'Hebron University Hospital, Barcelona, Spain; [4]Spanish Biomedical Research Network Centre in Oncology (CIBERONC), Madrid, Spain; [5]Medical Oncology Hospital Clínic, Barcelona, Spain; [6]Translational Genomics and Targeted Therapeutics Group, IDIBAPS, Barcelona, Spain; [7]Oncology Data Science (ODysSey Group), Vall d'Hebron Institute of Oncology, Barcelona, Spain; [8]Medical Oncology Hospital Vall d'Hebrón, Barcelona, Spain; [9]IOB Institute of Oncology, Quironsalud group, Quirón University Hospital, Barcelona, Spain; [10]IrsiCaixa, Hospital Universitari Trias i Pujol, Badalona, Spain; [11]Barcelona Supercomputing Center, Barcelona, Spain; [12]IOB Institute of Oncology, Quironsalud group, Madrid, Spain.

INTRODUCTION AND OBJETIVES

Triple negative breast cancer (TNBC) is a chemo-sensitive breast cancer subtype, but has an aggressive behavior with high rates of local and distance relapse. Several studies[1,2] have identified that high levels of tumor-infiltrating lymphocytes (TILs) at diagnosis of TNBC confers better prognosis and patients respond better to specific chemotherapies. But only 15% of TNBC patients have high levels of TILs, and another 15% has almost zero[3]. The aim of this study is to understand the mechanisms that impede immune infiltration in the tumor. Our main hypothesis is that lymphocytes are blocked in local lymph nodes by an immune checkpoint.

Primary objective: analyze 5 immune checkpoints in lymph nodes (LN) of patients with low TILs and compared to high TILs patients by immunohistochemistry (IHC).

Secondary objectives: a) analyze gene expression of 50 immune genes in LN with NanoString; b) analyze germinal centers (GC) and tertiary lymphoid structures (TLS) to study humoral response c) analyze, in the tumor, 5 biomarkers by IHC, 50 immune genes and the 360-breast cancer panel by NanoString, and mutation and neoantigen load by whole exome sequencing (WES).

PATIENTS AND METHODS

Inclusion criteria were T1c-T2N0M0 TNBC patients that did not have neoadjuvant treatment, and exclusion criteria were patients with intermediate TILs (5-50%) and unavailability of both LN or tumor. We included 35 patients [15 with high (≥50%) and 20 with low TILs (≤5%)] that underwent surgery between 1998 to 2009 (Figure 1). We followed the methodology of Figure 2 for the different tests performed.
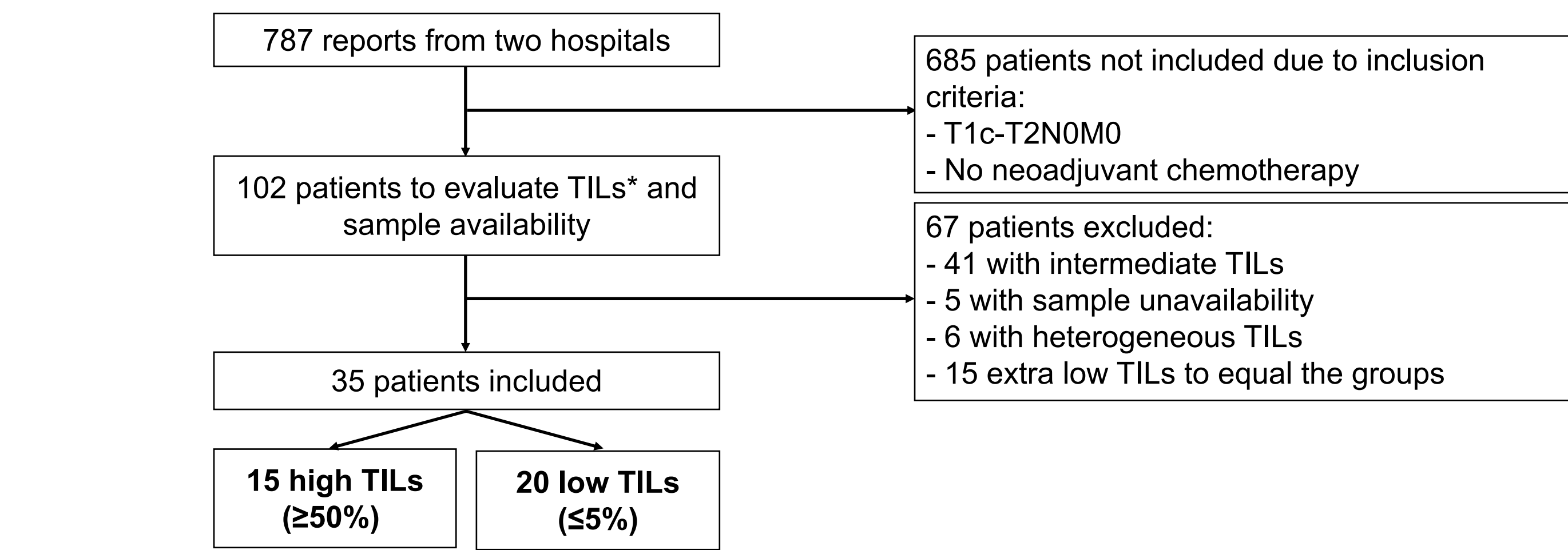

Figure 1. Flow diagram for the identification of patients and samples. *as per 2017 TIL evaluation guidelines[4].
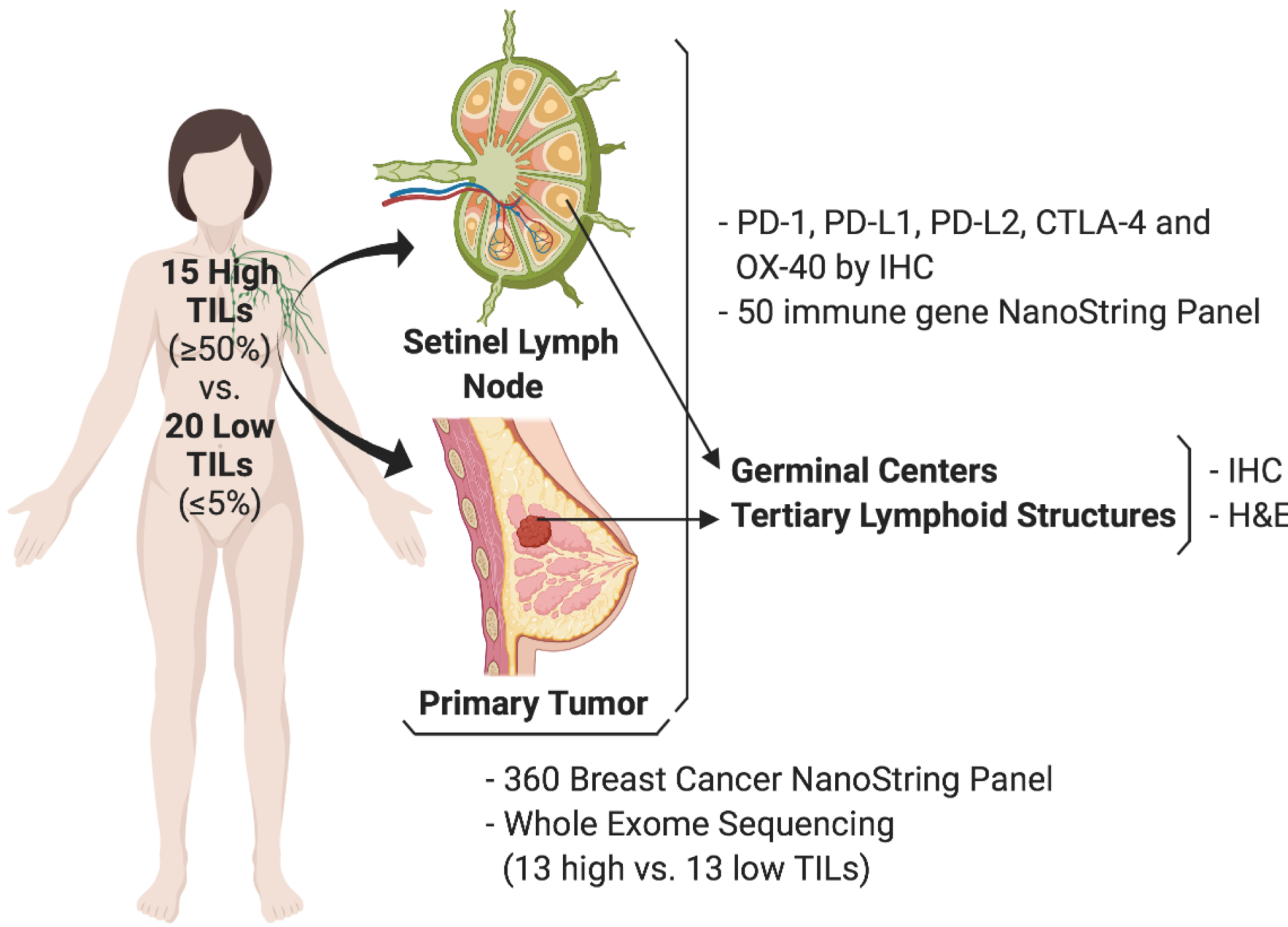

Figure 2. Methodology for the different tests performed. IHC: Immunohistochemistry; H&E: Hematoxylin and Eosin.

RESULTS

Table 1 provides a summary of patient's clinical and anatomopathological characteristics. The immune phenotype of high TIL samples was inflamed tumors; for low TILs we considered both the immune excluded and dessert phenotypes. By TNBC signature[5], 15/15 high TIL patients were BLIA (Basal-like Immune Activated), while low TILs were 9/18 (2 samples did not pass the quality control of NanoString).

Variables	High TILs (≥ 50%) 15 patients	Low TILs (≤ 5%) 20 patients
Age at diagnosis (years)		
- Mean	55	61
- Median	56	62
- Range	26 – 79	38 – 79
Type of surgery (%)		
- Tumorectomy	9 (60%)	16 (80%)
- Mastectomy	6 (40%)	4 (20%)
Tumor size (mm)		
- Mean	22,47	20,05
- Median	22	18
- Range	11 – 50	11 – 35
TNM (%)		
- T1cN0M0	6 (40%)	12 (60%)
- T2N0M0	9 (60%)	8 (40%)
Histology (%)		
- Invasive ductal G3	12 (80%)	15 (75%)
Lymph node resection (%)		
- Lymphadenectomy	9 (60%)	5 (25%)
- Sentinel Lymph/s Node/s Resection	6 (40%)	15 (75%)

Table 1. Patient's clinical and anatomopathological characteristics.

CTLA-4 is more expressed in lymphocytes from lymph nodes of low TIL patients

CTLA-4 and PD-1 stained lymphocytes very clear, the others stained several cells of the LN.
- CTLA-4 (p = 0.01, median 7% vs. 4%) and OX-40 (p < 0.01, median 30% vs. 15%) were more expressed in LN of low TIL patients by IHC. PD-1, PD-L1 and PD-L2 were not statistically significant (Figure 3).
- CD68 (t score 1.72) and CD8A (t score 1.28) were overexpressed in high TIL patients by SAM analysis on the 50-immune gene NanoString panel.
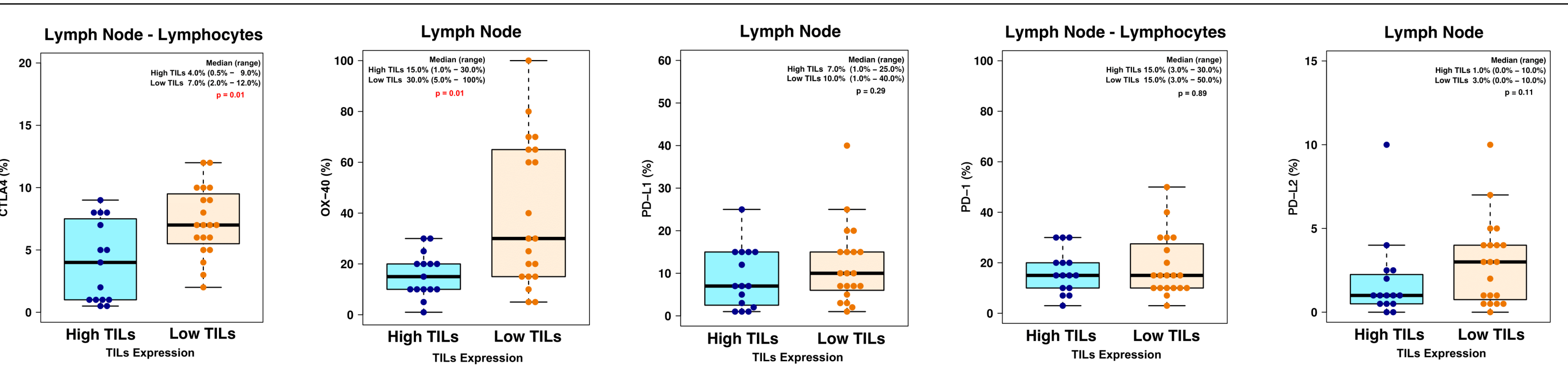

Figure 3. Box-plots comparing the expression of CTLA-4 (lymphocytes only), OX-40, PD-1 (lymphocytes only), PD-L1 and PD-L2 between lymph nodes of high and low TIL patients.

High TIL patients have more TLS and more and larger GC

We measured size and number of GC and TLS present in LN and tumors. We considered ≤3 GC as a limited humoral response and ≤100 μm (mean diameter) as a small GC.
- High TILs had more and larger GC (80% vs. 35%, p = 0.02) and
- had more TLS (47% vs. 25%, p = 0.28) than in low TIL patients.

High TIL patients have higher levels of PD-L1+ tumor cells but less neoantigens, low TILs overexpress B7-H3 and B7.H4

- PD-L1 was highly expressed in tumor cells of high TIL patients (p < 0.001, median 0.0% vs. 0.5%), and OX-40 in tumor cells of low TIL patients (p < 0.01, median 35% vs. 15%) (Figure 3A).
- PD-L1 and OX-40 usually stained stromal TILs adjacent to tumor, and coincided with the presence of CD8+ T cells (Figure 3B).
- B7.H4, B7.H3 (CD276), VEGF, CD73 and CD56 were overexpressed in low TILs by SAM analysis (p<0.05) and had more expression of stromal genes (Figure 3C and D).
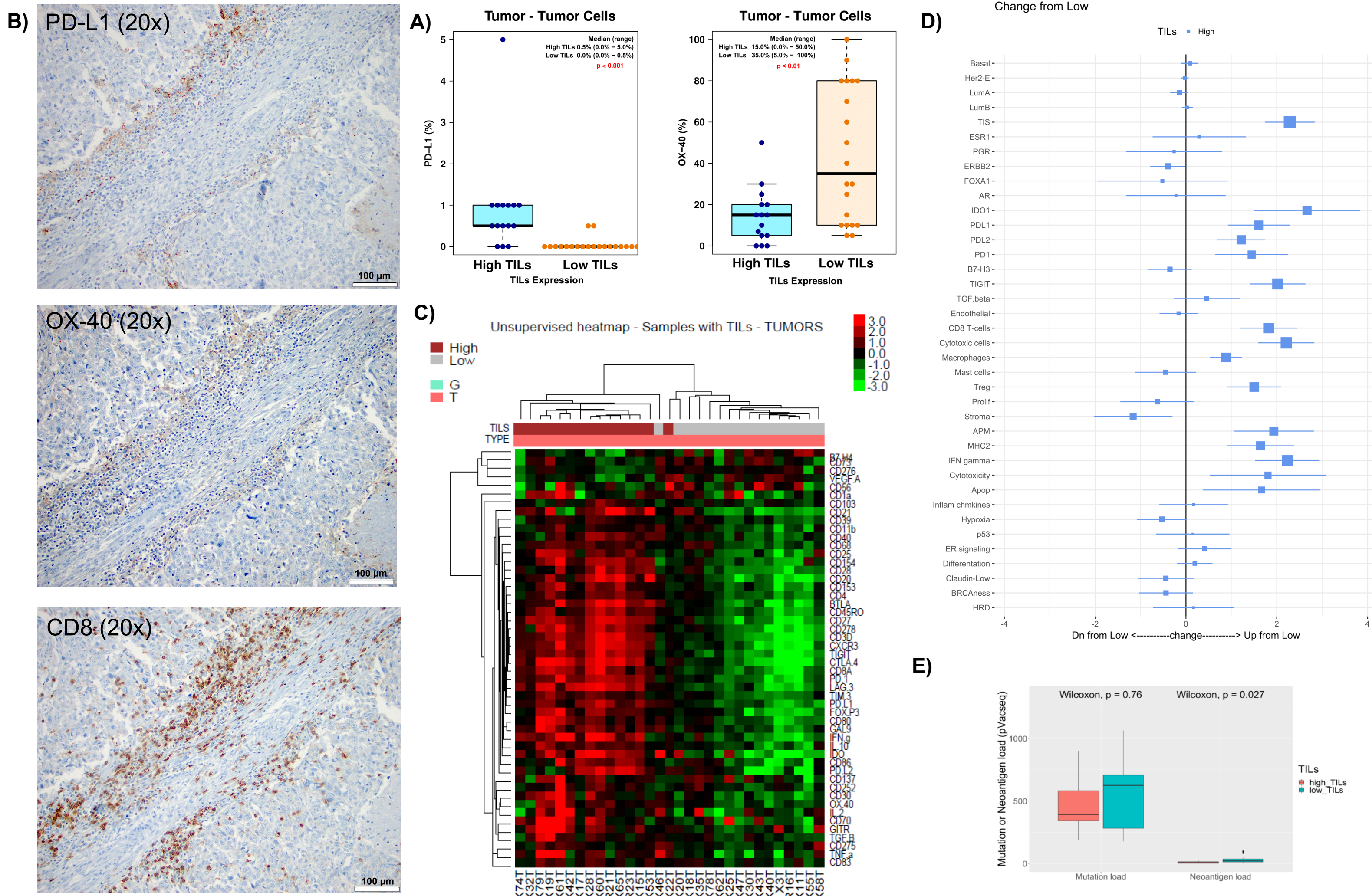- Low TILs had more neoantigen load (p = 0.027) (Figure 3E).

Figure 4. A) Box-plots comparing the expression of PD-L1 and OX-40 in tumor cells between primary tumor samples of high and low TIL patients. B) PD-L1, OX-40 and CD8 staining in the tumor of a high TIL patient; C) Heatmap of the 50-immune NanoString panel in tumor; D) Forest Plot of the 360 Breast Cancer NanoString panel; E) Mutation and neoantigen load comparison.

CONCLUSIONS

In low TIL patients, lymphocytes may be retained in lymph nodes by, at least, CTLA-4. They also have higher expression of B7.H3 and B7.H4 in the tumor. In high TIL patients, PD-L1 may act as a secondary brake in the tumor, and showed more developed humoral response and less neoantigens.

APPLICABILITY

We could apply anti-CTLA-4 treatments to increase immune infiltration in localized TNBC tumors with low TILs before surgery or neoadjuvant treatment, to improve their prognosis.

Further information: Javier Cortés (jacortes@vhio.net)

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