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Differences in the immune response of the non-metastatic axillary lymph nodes between triple-negative and luminal A breast cancer surrogate subtypes

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Abstract

Breast cancer (BC) comprises four immunohistochemical surrogate subtypes, of which triple-negative breast cancer (TNBC) has the highest risk of mortality. Axillary lymph nodes (ALNs) are the regions where BC cells first establish before distant metastasis, and the presence of tumor cells in the ALN gives rise to an immune tolerance profile that contrasts with that of the non-metastatic ALN (ALN^-). However, few studies have compared the immune components of the $ALNs^-$ in BC subtypes. The present study aimed to determine whether differences between immune populations in the primary tumor and $ALNs^-$ were associated with the luminal A or TNBC subtype. We evaluated a retrospective cohort of 144 patients using paraffin-embedded biopsies. TNBC samples tended to have a higher histological grade and proliferation index, and had higher levels of immune markers compared with luminal A in primary tumors and $ALNs^-$. Two methods for validating the multivariate analysis showed that histological grade, intratumoral S100 dendritic cells, and CD8 T lymphocytes and CD57 Natural Killers in the $ALNs^-$ were factors associated with TNBC, while CD83 dendritic cells in the $ALNs^-$ were associated with luminal A. In conclusion, we found that intratumoral regions and $ALNs^-$ of TNBC contained higher concentrations of markers related to immune tolerance than did Luminal A. This partially explains the worse prognosis of TNBC.

INTRODUCTION

Cancer is one of the most prominent of all human diseases, and breast cancer (BC) is the most frequent type in women.[1, 2] Four surrogate subtypes of BC, with distinct clinical behaviors, are recognized: luminal A-like, luminal B-like ($HER2^-$ and HER^+), $HER2^+$ (non-luminal) and triple-negative BC (TN).[3] These subtypes can be distinguished by immunohistochemistry (IHC), by quantifying the expression of estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2),[4, 5] and by determining tumor proliferation, measured by Ki67.[6] In particular, TNBC accounts for 10-15% of all BC cases and has a higher risk of mortality and worse prognosis than the other subtypes.[7, 8] In contrast, 60-70% of BC patients present the luminal A subtype, which has a better prognosis than the other subtypes and tends to relapse later than TNBC (after 5 years *vs.* within 2-3 years of first presentation, respectively).[9]

The microenvironment of the tumor, and especially that of the immune cell populations, plays a key role in BC progression and patient outcome. The immune system may play one of two roles, either defending the organism from cancer progression or stimulating tumoral growth, thereby facilitating the establishment and metastasis of cancer.[10, 11] This response is thought to differ depending on the BC subtype. For instance, TNBC in the primary tumor is more immunogenic than the luminal A subtype,[12, 13] and expresses a greater variety of cytokine receptors than do luminal BC cells as a consequence of its greater invasion and metastasis rates.[14]

In BC, as the primary tumor grows, it can invade nearby tissues and migrate into other regional or distant tissues or organs, where it establishes a new tumor or metastasis, the latter causing the majority of BC deaths. BC tumoral cells are known to escape the primary tumor through blood or lymphatic vessels.[15] In particular, axillary lymph nodes (ALNs) are one of the first regions where BC metastasis becomes established,[16] and its infiltration by tumoral cells is known to be a bad prognosis factor,[17, 18] which has been linked to the BC subtypes in various ways.[19] Although the ALN is particularly important in antitumoral immunity,[20] and the presence of tumor in the

ALNs gives rise to an immune tolerance profile,[21-23] little research has been done on the immune response in the ALNs, and less still on non-metastatic ALNs ($ALNs^-$). In a previous study, our group found the immune response of $ALNs^-$ to be an important factor in ALN metastasis in BC that could be indirectly involved in patient outcome.[24] Although the immune response is decisive in tumoral progression,[10, 11] few studies have compared the immune markers in the $ALNs^-$ from the TNBC and luminal A subtypes.

It is clear that the prognosis, ALN status and probability of metastatic spread of BC subtypes differ greatly. Moreover, the type of immune response is important in BC development, and the immune response in the ALN may be involved in tumor progression, even in the ALN^-. Given this evidence, we compared the expression of specific immune populations by IHC in BC patients diagnosed with luminal A and TNBC in the intratumoral microenvironment and in the ALN^-, and investigated their association with the BC subtype. To our knowledge, this is the first comparison of the immune response in the ALN^- of the two subtypes.

MATERIALS AND METHODS

Study design and participants

We evaluated a retrospective cohort of patients diagnosed with invasive breast carcinoma of no special type during the period between 2000 and 2008. Of the 144 BC patients evaluated, 88 were diagnosed as luminal A (61%) and 56 as TNBC (39%) subtypes. Regarding the treatments administered, 12.5% of Luminal A and 9.1% of TNBC patients received neoadjuvant therapy, 98.9% of luminal A and 100% of TNBC patients received adjuvant therapy (hormonal therapy and/or chemotherapy), and 80.7% of luminal A and 85.5% of TNBC patients received radiotherapy. We obtained biopsies from the primary tumors and $ALNs^-$ to evaluate 11 immune populations. The biopsies were collected from the Tumor Bank of the Pathology Department of the Hospital of Tortosa Verge de la Cinta (HTVC) and the Hospital Joan XXIII of Tarragona

(Spain). All the biopsies were reviewed using a new hematoxylin-eosin slide to check tissue availability.

The Ethics Committee of the Hospital Joan XXIII of Tarragona and the Research Committee of HTVC approved this study (reference number 24p/2012). All patients provided written informed consent to participate in the study and for their biopsies and clinical data to be used. We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

Tissue microarray construction and immunohistochemistry

Pathologists selected four representative areas to obtain four 2-mm cylinders: two from the primary tumor biopsies (intratumoral region), and two from the central region of ALN^- biopsies. This gave a total of 576 cylinders (352 from patients with luminal A subtype and 224 from patients with TNBC), which were incorporated into tissue microarrays (TMAs) as previously described.[25] Each TMA block contained 50 cylinders, so 12 TMAs were examined in total. Eleven slides were sectioned from each TMA, one for each of the 11 immune markers studied, producing a total of 132 slides. TMAs allow the evaluation of a large number of cases, but they have the drawback that they are not closely correlated enough with whole-tissue sections to permit clinical diagnosis. Even so, their use for research purposes is considered adequate,[26] and a PubMed search of TMAs in breast cancer yielded more than 200 articles, which is an indication of their popularity for studying the immune response in BC.[27, 28]

For the immunohistochemical detection in the immune cell populations studied here we used the following primary antibodies (Figure 1): T helper lymphocytes (anti-CD4, clone 4B12, Dako, Santa Clara, CA, USA), cytotoxic T lymphocytes (anti-CD8, clone C8/144B, Dako), natural killers (NK, anti-CD57, clone NK1, Zymed, Thermo Fisher Scientific, Waltham, MA, USA), regulatory T cells (anti-FOXP3, clone 236A/E7, CNIO, Madrid, Spain), macrophages (anti-CD68, clone KP1, Dako), follicular dendritic cells (DCs) (fDC, anti-CD21, clone 1F8, Dako), Langerhans DCs (anti-CD1a, clone 010, Dako), plasmacytoid DCs (anti-CD123, clone 6H6,

eBioscience, San Diego, CA, USA), interdigitant DCs (anti-S100, polyclonal, Leica Microsystems GmbH, Wetzlar, Germany), LAMP3 DCs (anti-CD208, polyclonal, Proteintech, Rosemont, IL, USA), and mature DCs (anti-CD83, clone1H4b, Leica Microsystems GmbH, Wetzlar, Germany). We carried out the final detection of the stainings using the ENDVISION™ FLEX method (Dako), with the chromogen diaminobenzidine (DAB, Dako) as a substrate and hematoxylin as counterstain, in accordance with manufacturer's protocol.

Slides were scanned at 40x magnification with the Aperio ScanScope XT scanner (Leica Microsystems GmbH, Nussloch, Germany). We obtained TIFF digital images at a resolution of 0.25 µm/pixel and with an approximate size of 25 GB. This method for obtaining these types of image is known as Whole Slide Imaging (WSI) and is the most recent imaging modality in the field of pathology.[29] To analyze images correctly, we examined each cylinder individually in a single image with automatic tools previously developed by our team.[30, 31] These 6,336 individual digital images (11 immune markers x 576 cylinders) were stored in TIFF format with a size of around 500 MB, and coded with the case number and name of the corresponding immune marker. Each cylinder area and its respective immune markers were evaluated by digital image analytical procedures, as previously described.[24, 25, 32-34]

The following clinical and pathological data were compiled: age, tumor diameter, number of metastatic lymph nodes, presence of axillar metastasis, lymphovascular invasion (LVI), perineural invasion (PNI), histological grade, proliferation index (Ki67), and menopausal status. These variables were compared in luminal A and TNBC patients.

Statistical analysis

To evaluate the differences in the mean or median concentrations of the studied immune populations and the differences in the quantitative clinical and pathological variables between the two BC subtypes, we used Student's independent-samples t-test or the Mann-Whitney U test. Categorical clinical and pathological variables were examined using the chi-squared or Fisher's exact test.

To assess which of the immunological, clinical and pathological variables were associated with each of the studied subtypes (taking luminal A as reference), univariate logistic regression models were fitted for each variable to estimate the odds ratio (OR) and 95% confidence interval (CI). These models allowed us to identify which of the studied variables were differentially associated with the TNBC and luminal A subtypes. We considered the variables attaining a level of significance of $p < 0.10$ in the univariate analyses to develop a multivariate logistic regression model. Model goodness of fit was concluded if the Hosmer–Lemeshow test was non-significant for all of the variables. Any quantitative variable that was significant in the univariate analysis that did contribute to the goodness of fit of the model was dichotomized according to its distribution: greater than or less than/equal to the mean (normally distributed variable) or median (non-normally distributed variable). As a rule of thumb, a logistic regression should be based on a minimum of ten events for each variable included in the model.[35, 36] Luminal A was used as the reference group in the analyses, with TNBC as the event. With a final multivariate model including 4 variables, the sample size of 57 events was therefore large enough. The ability of the model to correctly assign patients to the luminal A or TNBC subtype was evaluated using Nagelkerke's R^2, the sensitivity and specificity, and the receiver-operating characteristic (ROC) curve and its area under the curve (AUC).

The model obtained was validated by two statistical techniques. The first validation was performed with the bootstrapping simulation technique using IBM SPSS Statistics 23.0 (IBM, Armonk, NY, USA), assuming a 95% CI and using 10,000 samples. The second validation used the Multiple Imputation method of STATA 14.0 (StataCorp LLC, College Station, TX, USA). Ten imputed datasets were used to handle missing data, and all the potential predictive variables and outcomes were included in the imputation model. Rubin's rules were used to combine logistic model estimates and standard errors. The AUC was calculated for both validation methods. Values of $p < 0.05$ were considered statistically significant for all analyses.

RESULTS

Patients' clinical and pathological data

Table 1 summarizes the differences in the clinical and pathological characteristics between the luminal A and TNBC patients. As expected, we found that TNBC patients featured a significantly greater proportion of higher histological grades than the luminal A group. Following a similar pattern, the TNBC group also had a greater percentage of patients with a high level of Ki67.

Immune cell markers

The immune microenvironment of the intratumoral region in the TNBC group showed a statistically significantly higher concentration of the CD4, CD8, FOXP3, CD21, CD68, CD1a, CD123, S100 and CD208 markers than the luminal A subtype (Table 2). Nine of the eleven immune populations studied differed between subtypes with respect to concentrations in the intratumoral area. The $ALNs^-$ had similar results to those of the intratumoral region, with higher concentrations of CD4, CD8, CD57 and CD123 markers in the TNBC group. Conversely, CD83 tended to be present at significantly higher concentrations in the luminal A group than in the TNBC group.

Logistic regression

The associations of each variable with the tumor subtype estimated in univariate logistic regressions are presented in Table 3. To evaluate which of the variables were independently associated with each subtype, all the variables with an associated value of $p < 0.1$ in the univariate analyses were considered in the multivariate logistic regression model. The multivariate model (Table 3) indicated that none of the clinical or pathological factors was independently associated with the TNBC when the immunological variables were also considered in the analysis. On the other hand, regarding the immune populations, the model showed that higher concentrations of intratumoral S100, interdigitant DCs, CD8 cytotoxic T

lymphocytes, and CD57 NK cells in the ALNs$^-$ were factors independently associated with the TNBC subtype. CD83 mature DCs were also found to be a protective factor in the TNBC subtype. The Hosmer–Lemeshow test was not significant (p = 0.839), indicating a good fit.

Nagelkerke's R^2 for the model was 0.732, which means that about three-quarters of the variation between the subtypes may be explained by the immune response of the primary tumor and ALNs$^-$. The ROC curve (Figure 2) showed that the multivariate model had a sensitivity of 81.3% and a specificity of 91.0%. Its AUC was 0.956 (95% CI: 0.916-0.995).

Finally, we turn to the results of the two methods used to validate the multivariate model. First, the bootstrap simulation produced similar results to those of the unvalidated multivariate model. The S100 immune marker in the intratumoral region had an OR of 2.04 (95% CI: 1.10-3.79; p = 0.024). The ORs for the immune markers in the ALN$^-$ were: CD8, 1.15 (95% CI: 1.04-1.28; p = 0.002); CD57, 23.23 (95% CI: 4.76-113.30; p < 0.001); and CD83, OR=0.052 (95% CI: 0.012-0.23; p < 0.001). The Hosmer–Lemeshow test was not significant (p = 0.773), indicating a good fit. The AUC was 0.960 (95% CI: 0.960-0.961) (Figure 2) with a Nagelkerke's R^2 of 0.746. The validation of the multivariate model by multiple imputation confirmed the immune populations of the ALN$^-$ in the unvalidated model: CD8, OR = 1.12 (95% CI: 1.04-1.21; p = 0.004); CD57, OR = 15.40 (95% CI: 3.10-76.60; p = 0.001), fitted as a continuous variable since, after multiple imputation, the Hosmer–Lemeshow test showed it to have a good fit; and CD83, 0.19 (95% CI: 0.07-0.51; p < 0.001). The intratumoral S100 immune population was not significant and so was not included in the final model. On the other hand, histological grade was significant in the validated model: grade 2, OR = 2.01 (95% CI: 0.21-19.33; p = 0.547); and grade 3, 12.27 (95% CI: 1.39-108.17; p = 0.024). The Hosmer–Lemeshow test indicated a good fit (p = 0.466). The model had an AUC of 0.940 (95% CI: 0.898-0.982) (Figure 2) and a Nagelkerke's R^2 of 0.716. Thus, the significant associations in the multivariate model of the three immune populations with TNBC in the ALNs$^-$ were confirmed by both validation methods.

DISCUSSION

Most studies in this field have compared metastatic ALNs ($ALNs^+$) with primary tumor or with $ALNs^-$;[37] not many have compared the immune response in $ALNs^-$ of different types of BC patients.[24, 38] Thus, we evaluated the differences in immune cell markers in the intratumoral region and $ALNs^-$ of patients with luminal A and TNBC subtype invasive BCs. We found that the TNBC subtype had more aggressive features a higher histological grade and a higher proliferation index than luminal A as noted in earlier studies.[7, 39-41] Some studies have reported the metastatic status of the ALN to be more closely associated with the TNBC than the luminal A subtype.[39, 40, 42-48] Other authors have noted that TNBC cases have the same[49] or a lower[50] probability of developing ALN metastasis than other subtypes, but we did not find any significant differences in the proportion of ALN^+ between the two groups. Apart from the histological differences, the immune response in the tumor is also known to differ according to the BC subtype, since the TNBC subtype more strongly activates the immune system than does luminal A.[12] We found clear differences between the subtypes, whereby a significantly higher proportion of stained cells in nine of the eleven immune populations studied was present in the intratumoral region of the TNBC. Similar results were found in the $ALNs^-$ where four immune populations had a higher proportion of stained cells in the TNBC patients and one was at a higher proportion in the luminal A. Although fighting cancer is a primary function of the immune cells, cancer promotes immunosuppression, thus immune cells may shift their action towards tolerance to cancer.[51, 52] Six of the eleven studied markers that were highly expressed in TNBC samples could be directly or indirectly linked to tolerance to cancer. Several studies[53-57] have suggested that CD123 DCs and FOXP3+ cells stimulate tolerance of tumoral cells. Controversies surround the role of CD4 and CD8 in cancer progression but some studies have reported that they contribute to a worse outcome.[58-60] In addition, a high density of CD68+ tumor-associated macrophages and S100 expression have been linked to worse outcome.[61, 62] This is consistent with our findings and indicates that immune cells in TNBC may become tumor-tolerant and promote bad prognosis.

Considering the factors associated with each intrinsic subtype, we found that some immune cell populations in the intratumoral region and in the ALN^- were more strongly associated with the subtypes than the well-established pathological factors. We had previously noted this strong association when we studied which immune populations were associated with the occurrence of metastasis in the ALN.[24] Nevertheless, as previously discussed, this does not mean that immune cell populations are more important than the pathological factors used for predicting patient outcome, but they are complementary and could also be associated with patient outcome. This is evident from the multivariate model, in which we found immune populations to be associated only with the BC subtypes, while the pathological factors proved not to make a significant contribution. At the intratumoral level, only higher concentrations of interdigitant S100 DCs were associated with the TNBC subtype. These DCs are derived from bone marrow and located in the T cell domains of various humans tissues.[63] S100 gene expression is more active in the basal than non-basal BC subtype, and although S100 proteins are a diverse group, the expression of most of them is linked to worse outcomes.[62] Regarding the $ALNs^-$, higher concentrations of $CD8^+$ T lymphocytes and $CD57^+$ NK cells were significantly associated with the TNBC, while higher proportions of mature $CD83^+$ DCs were associated with the luminal A subtype. In the intratumoral area, tumoral cells directly affect the microenvironment, modulating the immune response, while metastasis has not yet been established in the $ALNs^-$, so the immune responses are expected to be different. Even so, some studies have provided evidence that the primary tumor alters the immune microenvironment in the ALNs even before establishing metastasis.[23, 38] Hu et al. suggested that NK (CD57) cells are associated with better clinical outcomes in a variety of solid tumors,[64] but they did not include BCs in their study. Although we cannot estimate overall survival or relapse-free survival, as explained before, TNBCs are typically more aggressive and have a worse prognosis than luminal A. This makes it difficult to explain the higher proportion of $CD57^+$ NK cells, but it might be related to the increased immunogenicity of TNBCs. Moreover, although the presence of $CD57^+$ NK cells indicates a possible better prognosis in solid tumors, the nature of their involvement in the $ALNs^-$ might not be the same. Indeed, Rezaeifard et al. recently showed that the presence of NK

lymphocytes in the tumor draining lymphatic nodes was associated with poor prognosis in BC patients.[65] Similarly, although controversy persists regarding the association of $CD8^+$ T lymphocytes with better or worse prognosis,[58, 59, 66-68] their presence in TNBC is usually associated with increased patient survival.[69, 70] Some authors have suggested that the differences between subtypes arise because highly proliferative cancers, such as TNBC, develop a stronger immune response.[70] Accumulation of $CD57^+$ and $CD8^+$ T cells occurs frequently in individuals with various forms of cancer and has been linked to reduced survival.[71-75] Our study demonstrated an association of $CD8^+$ T lymphocytes and $CD57^+$ NK cells in the $ALNs^-$ with the TNBC, possibly because of the greater proliferation of this subtype. This explains its stronger immunogenicity, but it could also arise because the $ALNs^-$ are not sufficiently affected by the immune escape component of the immunoediting process of tumoral growth by that stage. This means that neither the immune resistance of tumoral cells nor the downregulation of effector cells such as $CD8^+$ lymphocytes that typically occurs in advanced tumors may have affected the $ALNs^-$.[76] It should also be borne in mind that most of the studies of the immune response have focused on primary tumors, and so the role of these immune cells in the ALNs, in particular the $ALNs^-$, might differ.

The expression of mature $CD83^+$ DCs is regarded as an independent prognostic marker of survival,[77] which is consistent with the higher concentration we observed in the $ALNs^-$ of the luminal A subtype. Overall, our results suggest that the ALN^- is an active immunogenic region where the immune response to cancer develops. On the other hand, validating our multivariate model by multiple imputation led to the inclusion of histological grade as an independently significant factor. As previously explained, higher histological grade may be linked to the greater aggressivity of the TNBC,[7] although, in our study, the immune markers were closely associated with the BC subtypes. This shows that the BC subtype not only affects these clinical and pathological parameters, but also strongly affects the immune response, particularly in the $ALNs^-$. Moreover, S100 (in one of the two validations) in the intratumoral region, and the CD8, CD57 and CD83 markers in the $ALNs^-$ (in both validations) are independent factors associated

with the TNBC subtype, which suggests that they may be good predictors of the BC subtype. The great ability to predict the BC subtype clearly illustrates how the immune response in the ALNs$^-$ can be associated with each subtype.

In conclusion, our results show that the luminal A and TNBC subtypes clearly differ with respect to their proportions of immune markers, not only in the primary tumor, as expected, but also in the ALNs$^-$, an aspect that has not been studied before. TNBC has a more active immune response, with an emphasis on the markers related to immune tolerance, in the intratumoral region and in the ALNs$^-$, which might be related to its poorer prognosis and histological grade. These results are significant because they highlight the importance of the immune response of the ALNs$^-$ in cancer progression. We are aware of the limitations of IHC, one of which is that using single IHC markers for specific immune populations could lead to them being overestimated. Nevertheless, our results make it clear that differences exist in the composition of the immune cells in the ALNs$^-$. Further studies are required to fully establish the specific type of the immune cells in the ALN$^-$ and their role in the survival and relapse of BC patients.

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REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012, Int J Cancer 2015, 136:E359-386
2. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2020, CA Cancer J Clin 2020, 70:7-30
3. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, Senn HJ: Personalizing the treatment of women with early breast cancer: Highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2013, Ann Oncol 2013, 24:2206-2223
4. Hon JD, Singh B, Sahin A, Du G, Wang J, Wang VY, Deng FM, Zhang DY, Monaco ME, Lee P: Breast cancer molecular subtypes: From TNBC to QNBC, Am J Cancer Res 2016, 6:1864-1872
5. Cho N: Molecular subtypes and imaging phenotypes of breast cancer, Ultrasonography 2016, 35:281-288
6. Harbeck N, Gnant M: Breast cancer, Lancet 2017, 389:1134-1150
7. Foulkes WD, Smith IE, Reis-Filho JS: Triple-negative breast cancer, New Engl J Med 2010, 363:1938-1948
8. Li X, Yang J, Peng L, Sahin AA, Huo L, Ward KC, O'Regan R, Torres MA, Meisel JL: Triple-negative breast cancer has worse overall survival and cause-specific survival than non-triple-negative breast cancer, Breast Cancer Res Treat 2017, 161:279-287
9. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, Ruddy K, Tsang J, Cardoso F: Breast cancer, Nat Rev Dis Primers 2019, 5:66
10. Coleman RE, Gregory W, Marshall H, Wilson C, Holen I: The metastatic microenvironment of breast cancer: Clinical implications, Breast 2013, 22 Suppl 2:S50-56
11. Nagarajan D, McArdle SEB: Immune landscape of breast cancers, Biomedicines 2018, 6:20
12. Garcia-Teijido P, Cabal ML, Fernandez IP, Perez YF: Tumor-infiltrating lymphocytes in triple negative breast cancer: The future of immune targeting, Clin Med Insights Oncol 2016, 10:31-39
13. Liu Z, Li M, Jiang Z, Wang X: A comprehensive immunologic portrait of triple-negative breast cancer, Transl Oncol 2018, 11:311-329
14. Levano KS, Jung EH, Kenny PA: Breast cancer subtypes express distinct receptor repertoires for tumor-associated macrophage derived cytokines, Biochem Biophys Res Commun 2011, 411:107-110
15. Scully OJ, Bay BH, Yip G, Yu Y: Breast cancer metastasis, Cancer Genom Proteom 2012, 9:311-320
16. Valente AL, Kane JL, Ellsworth DL, Shriver CD, Ellsworth RE: Molecular response of the axillary lymph node microenvironment to metastatic colonization, Clin Exp Metastasis 2014, 31:565-572
17. Beenken SW, Urist MM, Zhang Y, Desmond R, Krontiras H, Medina H, Bland KI: Axillary lymph node status, but not tumor size, predicts locoregional recurrence and overall survival after mastectomy for breast cancer, Ann Surg 2003, 237:732-738; discussion 738-739
18. Lale Atahan I, Yildiz F, Ozyigit G, Sari S, Gurkaynak M, Selek U, Hayran M: Percent positive axillary lymph node metastasis predicts survival in patients with non-metastatic breast cancer, Acta Oncol 2008, 47:232-238
19. He ZY, Wu SG, Yang Q, Sun JY, Li FY, Lin Q, Lin HX: Breast cancer subtype is associated with axillary lymph node metastasis: A retrospective cohort study, Medicine 2015, 94:e2213
20. Kim R, Emi M, Tanabe K, Arihiro K: Immunobiology of the sentinel lymph node and its potential role for antitumour immunity, Lancet Oncol 2006, 7:1006-1016
21. Chang AY, Bhattacharya N, Mu J, Setiadi AF, Carcamo-Cavazos V, Lee GH, Simons DL, Yadegarynia S, Hemati K, Kapelner A, Ming Z, Krag DN, Schwartz EJ, Chen DZ, Lee PP: Spatial

organization of dendritic cells within tumor draining lymph nodes impacts clinical outcome in breast cancer patients, J Transl Med 2013, 11:242

22. Faghih Z, Erfani N, Haghshenas MR, Safaei A, Talei AR, Ghaderi A: Immune profiles of CD4+ lymphocyte subsets in breast cancer tumor draining lymph nodes, Immunol Lett 2014, 158:57-65

23. Mansfield AS, Heikkila PS, Vaara AT, von Smitten KA, Vakkila JM, Leidenius MH: Simultaneous Foxp3 and IDO expression is associated with sentinel lymph node metastases in breast cancer, BMC cancer 2009, 9:231

24. Lopez C, Bosch R, Orero G, Korzynska A, Garcia-Rojo M, Bueno G, Fernandez-Carrobles MDM, Gibert-Ramos A, Roszkowiak L, Callau C, Fontoura L, Salvado MT, Alvaro T, Jaen J, Roso-Llorach A, Llobera M, Gil J, Onyos M, Plancoulaine B, Baucells J, Lejeune M: The immune response in nonmetastatic axillary lymph nodes is associated with the presence of axillary metastasis and breast cancer patient outcome, Am J Pathol 2020, 190:660-673

25. Callau C, Lejeune M, Korzynska A, Garcia M, Bueno G, Bosch R, Jaen J, Orero G, Salvado T, Lopez C: Evaluation of cytokeratin-19 in breast cancer tissue samples: A comparison of automatic and manual evaluations of scanned tissue microarray cylinders, Biomed Eng Online 2015, 14 Suppl 2:S2

26. Pinder SE, Brown JP, Gillett C, Purdie CA, Speirs V, Thompson AM, Shaaban AM: The manufacture and assessment of tissue microarrays: Suggestions and criteria for analysis, with breast cancer as an example, J Clin Pathol 2013, 66:169-177

27. Pelekanou V, Villarroel-Espindola F, Schalper KA, Pusztai L, Rimm DL: CD68, CD163, and matrix metalloproteinase 9 (MMP-9) co-localization in breast tumor microenvironment predicts survival differently in ER-positive and -negative cancers, Breast Cancer Res 2018, 20:154

28. Muftah AA, Aleskandarany MA, Al-Kaabi MM, Sonbul SN, Diez-Rodriguez M, Nolan CC, Caldas C, Ellis IO, Rakha EA, Green AR: Ki67 expression in invasive breast cancer: The use of tissue microarrays compared with whole tissue sections, Breast Cancer Res Treat 2017, 164:341-348

29. Farahani N, Pantanowitz L: Overview of telepathology, Clin Lab Med 2016, 36:101-112

30. Fernandez-Carrobles Mdel M, Bueno G, Deniz O, Salido J, Garcia-Rojo M: Automatic handling of tissue microarray cores in high-dimensional microscopy images, IEEE J Biomed Health Inform 2014, 18:999-1007

31. Roszkowiak L, Lopez C: PATMA: Parser of archival tissue microarray, PeerJ 2016, 4:e2741

32. Lopez C, Lejeune M, Bosch R, Korzynska A, Garcia-Rojo M, Salvado MT, Alvaro T, Callau C, Roso A, Jaen J: Digital image analysis in breast cancer: an example of an automated methodology and the effects of image compression, Stud Health Technol Inform 2012, 179:155-171

33. Korzynska A, Neuman U, López C, Lejeune M, Bosch R: The method of immunohistochemical images standardization. Edited by Choras R. Berlin, Heidelberg, Springer, 2010, p. pp. 213-221

34. Korzynska A, Roszkowiak L, Zak J, Lejeune M, Orero G, Bosch R, López C: The METINUS Plus method for nuclei quantification in tissue microarrays of breast cancer and axillary node tissue section, Biomed Signal Process Control 2017, 32:1-9

35. Peduzzi P, Concato J, Kemper E, Holford TR, Feinstein AR: A simulation study of the number of events per variable in logistic regression analysis, J Clin Epidemiol 1996, 49:1373-1379

36. Vittinghoff E, McCulloch CE: Relaxing the rule of ten events per variable in logistic and Cox regression, Am J Epidemiol 2007, 165:710-718

37. Gibert-Ramos A, Lopez C, Bosch R, Fontoura L, Bueno G, Garcia-Rojo M, Berenguer M, Lejeune M: Immune response profile of primary tumour, sentinel and non-sentinel axillary

lymph nodes related to metastasis in breast cancer: An immunohistochemical point of view, Histochem Cell Biol 2019, 152:177-193
38. Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP: Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer, PLoS Med 2005, 2:e284
39. He M, Zhang JX, Jiang YZ, Chen YL, Yang HY, Tang LC, Shao ZM, Di GH: The lymph node ratio as an independent prognostic factor for node-positive triple-negative breast cancer, Oncotarget 2017, 8:44870-44880
40. Borges US, Costa-Silva DR, da Silva-Sampaio JP, Escorcio-Dourado CS, Conde AM, Jr., Campelo V, Gebrim LH, da Silva BB, Lopes-Costa PV: A comparative study of Ki-67 antigen expression between luminal A and triple-negative subtypes of breast cancer, Med Oncol 2017, 34:156
41. Stathopoulos GP, Malamos NA, Markopoulos C, Polychronis A, Armakolas A, Rigatos S, Yannopoulou A, Kaparelou M, Antoniou P: The role of Ki-67 in the proliferation and prognosis of breast cancer molecular classification subtypes, Anticancer Drugs 2014, 25:950-957
42. Houvenaeghel G, Sabatier R, Reyal F, Classe JM, Giard S, Charitansky H, Rouzier R, Faure C, Garbay JR, Darai E, Hudry D, Gimbergues P, Villet R, Lambaudie E: Axillary lymph node micrometastases decrease triple-negative early breast cancer survival, Br J Cancer 2016, 115:1024-1031
43. Mando P, Rizzo M, de la Puente CP, Maino M, Ponce C, Pombo MT, Amat M, Costanzo MV, Nervo A, Nadal J, Fabiano V, Loza J, Loza CM, Colo F, Reinaldo C: High histologic grade and high Ki-67 expression predict phenotypic alterations in node metastasis in primary breast cancers, J Breast Cancer 2017, 20:170-175
44. Van Calster B, Vanden Bempt I, Drijkoningen M, Pochet N, Cheng J, Van Huffel S, Hendrickx W, Decock J, Huang HJ, Leunen K, Amant F, Berteloot P, Paridaens R, Wildiers H, Van Limbergen E, Weltens C, Timmerman D, Van Gorp T, Smeets A, Van den Bogaert W, Vergote I, Christiaens MR, Neven P: Axillary lymph node status of operable breast cancers by combined steroid receptor and HER-2 status: Triple positive tumours are more likely lymph node positive, Breast Cancer Res Treat 2009, 113:181-187
45. Lee JH, Kim SH, Suh YJ, Shim BY, Kim HK: Predictors of axillary lymph node metastases (ALNM) in a Korean population with T1-2 breast carcinoma: Triple negative breast cancer has a high incidence of ALNM irrespective of the tumor size, Cancer Res Treat 2010, 42:30-36
46. Arvold ND, Taghian AG, Niemierko A, Abi Raad RF, Sreedhara M, Nguyen PL, Bellon JR, Wong JS, Smith BL, Harris JR: Age, breast cancer subtype approximation, and local recurrence after breast-conserving therapy, J Clin Oncol 2011, 29:3885-3891
47. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA: Triple-negative breast cancer: Clinical features and patterns of recurrence, Clin Cancer Res 2007, 13:4429-4434
48. Nguyen PL, Taghian AG, Katz MS, Niemierko A, Abi Raad RF, Boon WL, Bellon JR, Wong JS, Smith BL, Harris JR: Breast cancer subtype approximated by estrogen receptor, progesterone receptor, and HER-2 is associated with local and distant recurrence after breast-conserving therapy, J Clin Oncol 2008, 26:2373-2378
49. Haffty BG, Yang Q, Reiss M, Kearney T, Higgins SA, Weidhaas J, Harris L, Hait W, Toppmeyer D: Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer, J Clin Oncol 2006, 24:5652-5657
50. Gangi A, Mirocha J, Leong T, Giuliano AE: Triple-negative breast cancer is not associated with increased likelihood of nodal metastases, Ann Surg Oncol 2014, 21:4098-4103
51. Corthay A: Does the immune system naturally protect against cancer?, Front Immunol 2014, 5:197
52. Kim R, Emi M, Tanabe K: Cancer immunoediting from immune surveillance to immune escape, Immunology 2007, 121:1-14

53. Shou J, Zhang Z, Lai Y, Chen Z, Huang J: Worse outcome in breast cancer with higher tumor-infiltrating FOXP3+ Tregs: A systematic review and meta-analysis, BMC cancer 2016, 16:687
54. Roncador G, Brown PJ, Maestre L, Hue S, Martinez-Torrecuadrada JL, Ling KL, Pratap S, Toms C, Fox BC, Cerundolo V, Powrie F, Banham AH: Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level, Eur J Immunol 2005, 35:1681-1691
55. Sisirak V, Faget J, Gobert M, Goutagny N, Vey N, Treilleux I, Renaudineau S, Poyet G, Labidi-Galy SI, Goddard-Leon S, Durand I, Le Mercier I, Bajard A, Bachelot T, Puisieux A, Puisieux I, Blay JY, Menetrier-Caux C, Caux C, Bendriss-Vermare N: Impaired IFN-alpha production by plasmacytoid dendritic cells favors regulatory T-cell expansion that may contribute to breast cancer progression, Cancer Res 2012, 72:5188-5197
56. Treilleux I, Blay JY, Bendriss-Vermare N, Ray-Coquard I, Bachelot T, Guastalla JP, Bremond A, Goddard S, Pin JJ, Barthelemy-Dubois C, Lebecque S: Dendritic cell infiltration and prognosis of early stage breast cancer, Clin Cancer Res 2004, 10:7466-7474
57. Ito T, Yang M, Wang YH, Lande R, Gregorio J, Perng OA, Qin XF, Liu YJ, Gilliet M: Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand, J Exp Med 2007, 204:105-115
58. Catacchio I, Silvestris N, Scarpi E, Schirosi L, Scattone A, Mangia A: Intratumoral, rather than stromal, CD8+ T cells could be a potential negative prognostic marker in invasive breast cancer patients, Transl Oncol 2019, 12:585-595
59. Matkowski R, Gisterek I, Halon A, Lacko A, Szewczyk K, Staszek U, Pudelko M, Szynglarewicz B, Szelachowska J, Zolnierek A, Kornafel J: The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer, Anticancer Res 2009, 29:2445-2451
60. Macchetti AH, Marana HR, Silva JS, de Andrade JM, Ribeiro-Silva A, Bighetti S: Tumor-infiltrating CD4+ T lymphocytes in early breast cancer reflect lymph node involvement, Clinics 2006, 61:203-208
61. Yang J, Li X, Liu X, Liu Y: The role of tumor-associated macrophages in breast carcinoma invasion and metastasis, Int J Clin Exp Path 2015, 8:6656-6664
62. McKiernan E, McDermott EW, Evoy D, Crown J, Duffy MJ: The role of S100 genes in breast cancer progression, Tumour Biol 2011, 32:441-450
63. Bodey B, Siegel SE, Kaiser HE: Antigen presentation by dendritic cells and their significance in antineoplastic immunotherapy, In Vivo 2004, 18:81-100
64. Hu G, Wang S: Prognostic role of tumor-infiltrating CD57-positive lymphocytes in solid tumors: A meta-analysis, Oncotarget 2018, 9:8111-8119
65. Rezaeifard S, Safaei A, Talei A, Faghih Z, Erfani N: NK, NKT and invariant-NKT cells in tumor draining lymph nodes of patients with breast cancer, Iran J Immunol 2019, 16:291-298
66. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, Ellis IO, Green AR: Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer, J Clin Oncol 2011, 29:1949-1955
67. Rathore AS, Kumar S, Konwar R, Makker A, Negi MP, Goel MM: CD3+, CD4+ & CD8+ tumour infiltrating lymphocytes (TILs) are predictors of favourable survival outcome in infiltrating ductal carcinoma of breast, Indian J Med Res 2014, 140:361-369
68. Huang Y, Ma C, Zhang Q, Ye J, Wang F, Zhang Y, Hunborg P, Varvares MA, Hoft DF, Hsueh EC, Peng G: CD4+ and CD8+ T cells have opposing roles in breast cancer progression and outcome, Oncotarget 2015, 6:17462-17478
69. Matsumoto H, Thike AA, Li H, Yeong J, Koo SL, Dent RA, Tan PH, Iqbal J: Increased CD4 and CD8-positive T cell infiltrate signifies good prognosis in a subset of triple-negative breast cancer, Breast Cancer Res Treat 2016, 156:237-247
70. Oh E, Choi YL, Park T, Lee S, Nam SJ, Shin YK: A prognostic model for lymph node-negative breast cancer patients based on the integration of proliferation and immunity, Breast Cancer Res Treat 2012, 132:499-509

71. Characiejus D, Pasukoniene V, Kazlauskaite N, Valuckas KP, Petraitis T, Mauricas M, Den Otter W: Predictive value of CD8highCD57+ lymphocyte subset in interferon therapy of patients with renal cell carcinoma, Anticancer Res 2002, 22:3679-3683
72. Characiejus D, Pasukoniene V, Jonusauskaite R, Azlauskaite N, Aleknavicius E, Mauricas M, Otter WD: Peripheral blood CD8highCD57+ lymphocyte levels may predict outcome in melanoma patients treated with adjuvant interferon-alpha, Anticancer Res 2008, 28:1139-1142
73. Akagi J, Baba H: Prognostic value of CD57(+) T lymphocytes in the peripheral blood of patients with advanced gastric cancer, Int J Clin Oncol 2008, 13:528-535
74. Sze DM, Brown RD, Yuen E, Gibson J, Ho J, Raitakari M, Basten A, Joshua DE, Fazekas de St Groth B: Clonal cytotoxic T cells in myeloma, Leuk Lymphoma 2003, 44:1667-1674
75. Van den Hove LE, Vandenberghe P, Van Gool SW, Ceuppens JL, Demuynck H, Verhoef GE, Boogaerts MA: Peripheral blood lymphocyte subset shifts in patients with untreated hematological tumors: Evidence for systemic activation of the T cell compartment, Leuk Res 1998, 22:175-184
76. Schreiber RD, Old LJ, Smyth MJ: Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion, Science 2011, 331:1565-1570
77. Iwamoto M, Shinohara H, Miyamoto A, Okuzawa M, Mabuchi H, Nohara T, Gon G, Toyoda M, Tanigawa N: Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas, Int J Cancer 2003, 104:92-97

Figure Legends

Figure 1. Immunohistochemical staining patterns for the immune markers in formalin-fixed, paraffin-embedded sections (magnification 40X). Representative examples of membrane (CD4, CD8, CD21), cytoplasmic (CD68, CD123, CD208), membrane and/or cytoplasmic (CD57, CD1a, CD83), nuclear and/or cytoplasmic (S100), and nuclear (FOXP3) reactivity of the biomarkers.

Figure 2. Receiver-operating characteristic (ROC) curve for the multivariate logistic regression model prediction.

Table 1. Differences in the clinical and pathological variables between patients with luminal A and triple-negative molecular BC profiles.

	Luminal A (n=88)	**Triple-negative (n=56)**	**p**
Age (years)	61.1 (11.7)	58.7 (12.0)	0.234*
Tumor diameter (mm)	18.0 (12.0)	18.5 (19.5)	0.959†
Number of metastatic lymph nodes	1.0 (2.0)	0.0 (2.0)	0.625†
Axillar metastasis			
Positive	46 (52.3%)	26 (46.4%)	0.608‡
Negative	42 (47.7%)	30 (53.6%)	
LVI			
Present	42 (47.7%)	8 (50.0%)	1.000‡
Absent	46 (52.3%)	8 (50.0%)	
PNI			
Present	28 (31.8%)	5 (31.3%)	1.000‡
Absent	60 (68.2%)	11 (68.8%)	
Histological grade			
1	24 (27.3%)	3 (5.4%)	< 0.001‡
2	45 (51.1%)	10 (17.9%)	
3	19 (21.6%)	43 (76.8%)	
Ki67 grade			
Low	35 (39.8%)	26 (47.3%)	0.037‡
Medium	35 (39.8%)	11 (20.0%)	
High	18 (20.5%)	18 (32.7%)	
Menopausal status			

Pre-menopausal	13 (15.9%)	2 (15.4%)	1.000‡
Menopausal	69 (84.1%)	11 (84.6%)	

LVI = lymphovascular invasion. PNI = perineural invasion. Data are presented as the mean (standard deviation) for Student's independent-samples t-test*, the median (interquartile range) for the Mann-Whitney U test†, and the number of patients (percentage) in each category for the chi-squared or Fisher's exact test‡.

Table 2. Differences in the concentration of the immune populations in the intratumoral and ALN$^-$ regions between patients with luminal A and TNBC.

	Luminal A (n=88)	**Triple-negative (n=56)**	**p**
Intratumoral			
CD4	0.61 (1.11)	3.34 (7.15)	< 0.001†
CD8	1.06 (1.53)	1.54 (3.31)	0.050†
CD57	0.10 (0.33)	0.12 (0.24)	0.993†
FOXP3	0.06 (0.13)	0.17 (0.31)	< 0.001†
CD21	0.001 (0.027)	0.006 (0.017)	0.021†
CD68	2.63 (2.16)	3.69 (4.46)	< 0.001†
CD1a	0.09 (0.20)	0.22 (0.68)	0.009†
CD123	0.000 (0.000)	0.035 (0.099)	< 0.001†
S100	0.12 (0.29)	0.32 (0.62)	0.002†
CD208	0.02 (0.08)	0.07 (0.15)	0.015†
CD83	0.11 (0.18)	0.11 (0.26)	0.650†
ALN$^-$			
CD4	56.25 (13.30)	63.32 (14.24)	0.004*
CD8	13.07 (8.47)	19.45 (8.35)	< 0.001†
CD57	0.18 (0.23)	0.76 (0.82)	< 0.001†
FOXP3	1.99 (1.42)	2.12 (1.64)	0.927†
CD21	0.70 (1.76)	1.01 (1.17)	0.539†
CD68	9.32 (4.93)	9.91 (6.82)	0.225†
CD1a	1.64 (3.49)	1.76 (3.19)	0.797†
CD123	1.32 (1.84)	2.35 (3.13)	0.001†
S100	4.16 (5.40)	3.95 (4.48)	0.621†

CD208	0.24 (0.41)	0.18 (0.27)	0.112†
CD83	1.07 (1.26)	0.19 (0.59)	< 0.001†

ALN- = non-metastatic axillary lymph node. Data presented are the mean (standard derivation) and the median (interquartile range), of the percentage of positive stained area expressed for each marker. Probabilities are those corresponding to Student's independent-samples t-test* or the Mann-Whitney U test†.

Table 3. Univariate and multivariate analyses of the differences in variables in TNBC compared with luminal A subtype.

	Univariate OR (95% CI)	p	Multivariate OR (95% CI)	p
Age (years)	0.98 (0.95-1.01)	0.233		
Tumor diameter (mm)	1.00 (0.98-1.02)	0.761		
Number of metastatic lymph nodes	1.01 (0.94-1.08)	0.837		
Axillar metastasis				
Positive	0.79 (0.40-1.55)	0.494		
Negative	1.0			
LVI				
Present	1.10 (0.38-3.18)	0.867		
Absent	1.0			
PNI				
Present	0.97 (0.31-3.07)	0.964		
Absent	1.0			
Histological grade				
3	18.11 (4.86-67.51)	< 0.001		
2	1.78 (0.45-7.08)	0.415		
1	1.0			
PI (Ki67)				
High	1.35 (0.59-3.08)	0.481		
Medium	0.42 (0.18-0.99)	0.046		
Low	1.0			
Menopausal status				

Post-menopausal	1.04 (0.21-5.23)	0.966		
Pre-menopausal	1.0			
Intratumoral				
CD4	1.26 (1.12-1.41)	< 0.001		
CD8	1.07 (0.97-1.18)	0.181		
CD57	1.00 (0.94-1.06)	0.886		
FOXP3	35.60 (4.79-264.68)	0.001		
CD21c				
> median	2.50 (1.24-5.05)	0.011		
≤ median	1.0			
CD68	1.14 (1.01-1.30)	0.034		
CD1a	1.82 (1.06-3.14)	0.030		
CD123c				
> median	11.55 (5.12-26.06)	< 0.001		
≤ median	1.0			
S100	1.93 (1.01-3.70)	0.046	2.03 (1.09-3.77)	0.026
CD208				
> median	3.45(1.65-7.19)	0.001		
≤ median				
CD83	3.50 (0.97-12.63)	0.056		
ALN$^-$				
CD4	1.04 (1.01-1.07)	0.005		
CD8	1.09 (1.03-1.15)	0.001	1.15 (1.03-1.28)	0.011
CD57c				
> median	10.82 (4.69-24.92)	< 0.001	22.65 (4.63-110.73)	< 0.001

≤ median	1.0		1.0	
FOXP3	1.06 (0.79-1.43)	0.685		
CD21	0.96 (0.80-1.15)	0.642		
CD68	1.06 (0.98-1.14)	0.151		
CD1a	1.03 (0.93-1.14)	0.541		
CD123	1.48 (1.17-1.86)	0.001		
S100	0.98 (0.89-1.09)	0.752		
CD208	0.42 (0.14-1.24)	0.116		
CD83	0.15 (0.07-0.32)	< 0.001	0.053 (0.012-0.239)	< 0.001