Exploring the lymph node’s microenvironment for personalized management of Luminal A breast cancer

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Abstract

Background

Lymph nodes (LNs) are the main doorway for tumor cell metastases from the primary site and its evaluation is a major prognostic factor. The One Step Nucleic Acid Amplification (OSNA) is being adopted worldwide for sentinel-LNs (SLNs) staging in breast cancer (BC). SLNs’ OSNA lysate may be used for gene expression studies, being the potentially ideal samples to search for new markers related to immune response. Using a targeted gene expression approach, we aim to identify transcriptomic patterns of SLNs immune response and biomarkers that may improve risk stratification and personalized therapy for patients with Luminal A BC.

Methods

This was an observational, prospective, pilot study that included 32 patients with Luminal A early-stage BC: 16 patients with OSNA negative SLNs and 16 patients with OSNA positive SLNs. After the OSNA assay, rather than being discarded, the remaining OSNA lysates were prepared for target RNA sequencing (RNA-seq) analysis, using the Oncomine™ Immune Response Research Assay. Identification of differentially expressed genes (DEGs) for group comparisons was performed by DESeq2 R package (version 1.36.0) in R (version 4.2.0). Data analysis was performed using STATA software, version 13.1, and statistical significance was set at p < 0.05.

Results

In Luminal A BC patients, several genes were upregulated in metastatic SLNs, including KRT7, VTCN1, CD44, GATA3, ALOX15B, RORC and NECTIN2. In macrometastatic SLNs, LRG1, CD276, FOXM1 and IGF1R were also upregulated. In metastatic SLNs, higher values of total tumor load (TTL) correlated with a higher expression of most DEGs. Hierarchical clustering analysis revealed three different clusters, not coincident with pN0, pN1mi and pN1 classification.

Conclusions

A better understanding of the complex interplay between cancer cells and host immunity is essential for the choice of personalized treatments in Luminal A BC. The identified DEGs codify proteins mainly involved in cancer aggressiveness and with impact in immune response. These DEGs may improve prognosis accuracy and increase the efficacy and safety of targeted therapies in Luminal A BC patients. As OSNA assay is being implemented for SLNs staging in other cancers, RNA-seq in the OSNA lysate could also have a wider utility.

1. Introduction

Lymph nodes (LNs) are the main doorway for tumor cell metastases and its evaluation is a major prognostic factor. Two-thirds of breast cancer (BC) patients diagnosed with LNs metastases will develop distant metastases and 73% of these women will be dead within 5 years after diagnosis.(1, 2) SLN biopsy is the standard approach for loco-regional staging in patients with clinically T1-T2 invasive BC presenting with a clinically negative axilla.(3, 4) Patients with negative SLNs require no further axillary surgery. (5, 6) Patients with 1 or 2 metastatic SLNs who meet the criteria of ACOSOG Z0011 or AMAROS trials, completion of axillary LN dissection (ALND) is not necessary if irradiation and systemic adjuvant therapy are planned.(3, 7–10) However, currently, for Luminal node-positive BC, it is recommended an extension of endocrine therapy towards a duration of 10 years based on persistent risks of recurrence among such patients.(5, 6, 11)

Conventional intraoperative histological examinations of SLNs’ frozen sections are associated with 10–30% false-negative results for metastases.(12) Nevertheless, despite serial step section examination of each SLN being possible to overcome the false-negative results, it would be impractical because it requires a heavy workload for pathologists.(12) To overcome this issue, a
molecular method, the One Step Nucleic Acid Amplification (OSNA), based on reverse transcription loop mediated isothermal amplification (RT-LAMP) of cytokeratin 19 (CK19) mRNA in the lysate of SLNs, is being adopted worldwide by an increasing number of BC care centers. OSNA has several advantages: allows the analysis of the whole SLN, is semi-quantitative, standardized, reproducible, quicker (30 to 40 minutes from the excision of the SLN) and also diminish the pathologist workload. The OSNA cut-off levels were determined by Tsujimoto et al: macrometastases was defined as > 5,000 copies/µL of CK19 mRNA, micrometastases as 250 to 5,000 copies/µL and a value < 250 copies/µL correspond to absence of metastases or presence of isolated tumor cells (ITC). Total tumor load (TTL) was defined as the sum of the total number of CK19 mRNA copies in all positive SLNs (in copies/µL). Previous studies revealed that TTL is an independent predictor of the status of the non-sentinel LNs in BC patients and to be independently correlated with disease free survival, local recurrence free survival and overall survival. Nevertheless, the exact TTL cut-off to determine ALND is still under debate. Although LNs metastases is among the strongest predictors of prognosis, few studies have focused on the assessment of immunoinflammatory response in the LNs and the mechanisms that underlie the local failure of effective anti-tumor immune responses remain poorly understood. In LNs, exposure to tumor-derived factors induces stromal reprogramming, modifies immune cell population dynamics and affects chemokines and interleukins levels, which have the potential to contribute to impaired immune system response. Metastatic LNs are associated with an increased number of plasmacytoid dendritic cells (DCs), regulatory T lymphocytes (Tregs), immature DCs, higher expression of CD163+ M2 macrophages and a lower activation of CD8+ cytotoxic T lymphocytes, suggesting a deficient immune response. On the other hand, CD1a DC, mature DC, or CD169+ M1 macrophages are increased in the primary tumor and LNs of patients with non-metastatic LNs, suggesting a more efficient immune response. Also, on metastatic SLNs, expression of CD83, IL-12p40, IFN-γ, IL-10, and FOXP3 is higher than in non-metastatic SLNs. Thus, prognosis seems to depend not only on whether a patient has LNs metastases or not, but also on the type of local immune-cell population.

Different microenvironments were recognized in each subtype of BC and, consequently, specific microenvironments might be associated with distinct behaviors of the tumor cells and distinct prognosis and potential therapeutic targets. Studies on tumor microenvironment (TME) in patients with Luminal [estrogen receptors (ER) positive] HER2 negative BC are scarce, probably due to the good global survival rates (92.5% at 4 years) and the efficacy of the already existing therapies. Nevertheless, in Luminal HER2 negative BC, 31% had LN metastases and, for this group, the survival rates are lower (84.4% at 4 years). Considering that about 73% of BC are Luminal HER2 negative, this demonstrates the enormous untapped potential for immuno-targeted therapy in these patients.

As in OSNA assay most of the lysate sample is spared, it can be used for gene expression studies, evaluating microenvironment related genes, being the potentially ideal samples to search for new markers related to the immune SLNs response.

Using a next generation sequencing (NGS) targeted gene expression approach, we aim to identify transcriptomic patterns of immune response, at the level of SLNs, that may improve risk stratification and the development of targeted therapies for patients with Luminal A early BC.

2. Material And Methods

2.1. STUDY DESIGN AND PARTICIPANTS

This study was an investigator’s initiative, observational, prospective, pilot study. The project was approved by the Ethics Committee of Coimbra Hospital and University Centre (CHUC) (ethics approval number: CHUC-045-20). The project and the informed consent were written according to Good Clinical Practice and the Declaration of Helsinki and the samples were anonymized.

Patients with Luminal A early-stage BC (cT1-T2 N0) were invited to participate. The intrinsic subtype classification was based on international guidelines, and it was considered Luminal A BC if ER-positive, HER2-negative, Ki67 < 20% and PR ≥ 20%. Patients were enrolled until, consecutively, obtain 16 patients with OSNA negative SLNs and 16 with OSNA positive SLNs.
Inclusion criteria were defined including women with invasive BC, Luminal A subtype, cT1-2, cN0, surgical treatment including SLN biopsy and SLN analyzed by OSNA assay. As exclusion criteria, authors defined male BC, age under 18 years-old, pregnancy, germline mutations associated with breast hereditary cancer, neoadjuvant treatment, cytology proven LN metastases, distant metastases, tumors not expressing CK19, patients unable to give informed consent and technical limitations to SLN biopsy.

2.2. SLN BIOPSY AND OSNA ASSAY

SLNs were identified under combined techniques, using patent blue and radioisotope or superparamagnetic iron oxide, as previously described.(35, 36) After identification by the surgeon, SLNs were removed and directly sent to Pathology Department. The detailed OSNA assay has also been previously described.(14, 18, 37) In the Pathology Department, after the extra nodal tissue being removed, SLNs that exceeded the specified maximum weight (600mg) were cut into two or more pieces and processed as separate samples. Then, fresh SLNs were homogenized in 4 ml of a mRNA-stabilizing solution (Lynorhag® solution, Sysmex Corporation) using a RP-10 system (Sysmex Corporation; 90 seconds at 12 000 rpm). The homogenate (1 ml) was centrifuged for 1 minute at 12 200 x g and the intermediate phase was collected. A volume of 20 µl of the intermediate phase was used for the OSNA assay using the LYNOAMP™ CK19 (Sysmex Corporation) on the RD-210 system (Sysmex Corporation). A standard positive control sample and a negative control sample were used in every assay. Lastly, instead of being discarded, the remaining homogenate was kept at -80°C for RNA sequencing (RNA-seq) analysis.

The OSNA assay results were based on the calculated number of CK19 mRNA copies/µL, in accordance with the previous cut-off levels: > 5 000 copies/µL corresponding to macrometastases (pN1), 250 to 5 000 copies/µL to micrometastases (pN1mi), and values < 250 copies/µL were classified as negative SLN.(14) In negative SLNs, using RD-210 system (Sysmex Corporation), 160 to 249 corresponded to ITCs [pN0(i+)] and < 160 to absence of metastases (pN0).(38) TTL is defined as the sum of the numbers of CK19 mRNA copies in all positive SLNs.(13, 18)

The study had two major branches for SLN microenvironment analysis: 16 patients with OSNA positive SLNs and 16 patients with OSNA negative pN0 SLNs. The OSNA positive group was subdivided in SLNs with micrometastases and SLNs with macrometastases. Whenever more than one sample of the SLN or more than one SLN were diagnosed as having metastases, the one with a higher number of copies of CK19 mRNA/µL was considered for gene expression studies.

2.3. ALND: NON-SENTINEL LNs

The decision to proceed with ALND was discussed in a multidisciplinary team for each patient. Typically, ALND was performed in patients with metastatic SLNs with > 15 000 copies/µL of CK19 mRNA or if 3 or more positive SLNs were detected.(4, 5, 18) Non-sentinel LNs were assessed by current histological and immunohistochemical methods.

2.4. PATHOLOGICAL EVALUATION OF THE TUMOR

Parameters recorded by the pathologists were tumor’s larger diameter, presence of multicentricity or multifocality, LVI, histologic type, tumor grade, ER status, PR status, HER2 status, Ki-67 status, molecular classification and tumor infiltrating lymphocytes (TILs).(39–41) Stromal TILs were quantified on hematoxylin and eosin sections of tumor according to the guidelines of the International TILs Working Group.(41) The stromal TILs count was categorized into three grades: low (0-10%), intermediate (10-40%), or high (40-90%).(41, 42)

2.5. RNA SEQUENCING

Targeted RNA-seq and respective bioinformatics analysis was performed using the Oncomine™ Immune Response Research Assay – Chef-Ready library preparation kit (Thermo Fisher Scientific, Carlsbad, CA, USA). The Oncomine™ Immune Response Research Assay evaluates the expression of 395 genes, spanning across 36 functional groups, mainly associated with TME interplay (Additional file 1).(43)

For total RNA extraction, OSNA remaining homogenates were centrifuged for 1 min at 16 350 x g and 25 µl of the intermediate phase were used for RNA extraction applying the RNeasy® Plus Mini Kit (Qiagen GmbH, Hilden Germany), according to manufacturer instructions. DNA decontamination of RNA samples were confirmed with two polymerase chain reactions (PCRs). RNA concentrations were determined with the Qubit™ RNA HS Assay Kit and RNA integrity and quality were assessed with Qubit™
RNA IQ Assay, both using Qubit™ 4 Fluorometer (all from Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed with 10 ng of total RNA using Ion Torrent™ NGS Reverse Transcription Kit (Thermo Fisher Scientific) and a CFX96 thermocycler (Bio-Rad). cDNA (10 µl) was immediately used for automated library preparation with AmpliSeq™ Library Preparation reagents on an Ion Chef™ System (Thermo Fisher Scientific) (20 amplification cycles and 4 minutes of annealing and extension time). Libraries were quantified by real-time PCR with Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) in a CFX96 thermocycler (Bio-Rad). Template preparation and chip loading were automatically performed in the Ion Chef™ System using 25 µl of the 50 pM diluted library, Ion 530 Chef Kit and Ion 530 Chip (all from Thermo Fisher Scientific). The sequencing step was performed on the Ion GeneStudio™ S5 Plus System (Thermo Fisher Scientific, Waltham, MA, USA). RNA-seq analysis was obtained using Torrent Suite™ Software 5.16 and Immune Response Torrent Suite™ Plug-in.

2.6. STATISTICAL ANALYSIS

The database is blinded relative to patient identification. The calculations regarding patients, tumors and LNs characteristics were performed with the STATA software, version 13.1. The normal distribution of quantitative variables was evaluated through the Shapiro–Wilk test. Quantitative variables were described with minimum, maximum and mean [± standard deviation (SD)], while categorical variables were described as percentages. Moreover, the following statistical tests were applied when appropriate: Wilcoxon rank-sum test, two sample t-test, Kruskall-Wallis test, one-way ANOVA, simple linear regression and multiple linear regression for continuous variables; Fisher exact test, or chi-square test for categorical variables. Statistical significance was set at p < 0.05.

Principal component analysis (PCA) was performed by DESeq2 R package (version 1.36.0), using the complete set of genes, along the first two principal components. Identification of differentially expressed genes (DEGs) for different group comparisons was performed by DESeq2 R package (version 1.36.0) in R (version 4.2.0), using an adjusted p-value [False Discovery Rate (FDR)] threshold of 0.05 to correct for multiple hypothesis testing. The log2 fold cut-off for DEGs was set to changes greater than the absolute value of 0.58, corresponding to a fold change absolute value of 1.5. Hierarchical clustering heatmaps were performed in a data subset, comprising only significant DEGs, using the Pheatmap R package (version 1.0.12). The Euclidean distance was used to cluster both genes and samples. The unsupervised methods used DESeq2 regularized logarithm data transformation (rlog) as input.

Subsequent statistical analysis used gene expression data normalized by the median of ratios method using the DESeq2 R package. Two sample t-test, Wilcoxon rank-sum test, one-way ANOVA, Kruskal-Wallis test, Spearman correlation and linear regression were used to assess the relationship between normalized gene expression and clinical features.

3. Results

3.1. Clinicopathologic results

The clinical features of the 32 patients with Luminal A invasive BC included in this study are presented in Table 1, comparing the 16 patients with OSNA negative SLNs (pN0) and the 16 patients with OSNA positive SLNs (pN1 and pN1mi). There were no statistically significant differences related to clinical characteristics (Table 1).
Table 1

Clinical characteristics of the study group, comparing patients with OSNA negative SLNs (pN0) and patients with OSNA positive SLNs (pN1 and pN1mi).

|                       | OSNA negative | OSNA positive | p-value |
|-----------------------|---------------|---------------|---------|
| **Age, years**        |               |               |         |
| Minimum               | 48            | 43            | -       |
| Maximum               | 78            | 73            | -       |
| Mean ± SD             | 58.4 ± 7.8    | 58.1 ± 8.4    | 0.552   |
| **BMI, kg/m²**        |               |               |         |
| Minimum               | 18.3          | 16.6          | -       |
| Maximum               | 33.7          | 36.3          | -       |
| Mean ± SD             | 25.5 ± 3.5    | 27.0 ± 4.5    | 0.148   |
| BMI ≥ 30 kg/m² (%)    | 6.3% (n = 1)  | 18.8% (n = 3) | 0.300   |
| **Gravidity**         |               |               |         |
| Minimum               | 0             | 0             | -       |
| Maximum               | 5             | 4             | -       |
| Mean ± SD             | 2.1 ± 1.5     | 2.0 ± 1.1     | 0.554   |
| **Parity**            |               |               |         |
| Minimum               | 0             | 0             | -       |
| Maximum               | 3             | 3             | -       |
| Mean ± SD             | 1.8 ± 1.1     | 1.7 ± 0.9     | 0.569   |
| **Breastfeeding (%)** | 81.3% (n = 13)| 50.0% (n = 8) | 0.068   |
| **Breastfeeding, months** |         |               |         |
| Minimum               | 0             | 0             | -       |
| Maximum               | 48            | 24            | -       |
| Mean ± SD             | 12.2 ± 16.0   | 8.9 ± 10.4    | 0.529   |
| **Postmenopausal (%)**| 87.5% (n = 14)| 81.3% (n = 13)| 0.500   |
| **Age of menopause, years** |     |               |         |
| Minimum               | 45            | 45            | -       |
| Maximum               | 57            | 56            | -       |
| Mean ± SD             | 51.5 ± 3.7    | 50.8 ± 3.5    | 0.705   |
| **Smoker (%)**        | 18.8% (n = 3) | 13.2% (n = 2) | 0.532   |

SD – Standard deviation; BMI - Body Mass Index; ¥ - Two sample t-test; ¶ - Two-sided Chi-squared; Φ - Fisher’s exact test; £ - Wilcoxon rank-sum (Mann-Whitney) test
Regarding the histological characteristics of the tumor, the majority were No Special Type (NST) and had a single tumoral focus (Table 2). The OSNA positive group had a higher percentage of LVI, a higher grade and a higher Ki67 (Table 2). There were no statistically significant differences concerning the TILs (Table 2).
Table 2
Histological characteristics of the tumors, comparing patients with OSNA negative SLNs (pN0) and patients with OSNA positive SLNs (pN1 and pN1mi).

|                             | OSNA negative | OSNA positive | p-value |
|-----------------------------|---------------|---------------|---------|
| **Type of breast surgery (%)** |               |               |         |
| Partial mastectomy          | 100.0% (n = 16) | 87.5% (n = 14) | 0.484 φ |
| Total mastectomy            | 0.0% (n = 0)   | 12.5% (n = 2)  |         |
| **Histologic type (%)**     |               |               |         |
| No special type (NST)       | 75.0% (n = 12) | 93.8% (n = 15) | 0.311 φ |
| Lobular                     | 18.8% (n = 3)  | 6.3% (n = 1)   |         |
| Tubular                     | 6.3% (n = 1)   | 0.0% (n = 0)   |         |
| **Tumor diameter, mm**      |               |               |         |
| Minimum                     | 2.0           | 5.5           | -       |
| Maximum                     | 25.0          | 35.0          | -       |
| Mean ± SD                   | 14.1 ± 6.2    | 16.5 ± 7.6    | 0.173 ¥ |
| **Multifocality or multicentricity (%)** |           |               |         |
| 12.5% (n = 2)               | 25.0% (n = 4)  | 0.327 φ       |         |
| **Lymphovascular invasion (%)** |     |               |         |
| 6.3% (n = 1)                | 43.8% (n = 7)  | 0.019 φ       |         |
| **Grade**                   |               |               |         |
| Grade 1 (%)                 | 75.0% (n = 12) | 31.3% (n = 5)  | 0.020 φ |
| Grade 2 (%)                 | 18.8% (n = 3)  | 56.2% (n = 9)  |         |
| Grade 3 (%)                 | 0.0% (n = 0)   | 12.5% (n = 2)  |         |
| Unknown                     | 6.3% (n = 1)   | 0.0% (n = 0)   |         |
| Mean ± SD                   | 1.2 ± 0.4     | 1.8 ± 0.7     | 0.006 £ |
| **ER, %**                   |               |               |         |
| Minimum                     | 75            | 80            | -       |
| Maximum                     | 100           | 100           | -       |
| Mean ± SD                   | 91.6 ± 8.7    | 96.3 ± 6.5    | 0.081 £ |
| **PR, %**                   |               |               |         |
| Minimum                     | 20            | 25            | -       |
| Maximum                     | 100           | 100           | -       |
| Mean ± SD                   | 63.8 ± 26.8   | 75.0 ± 21.7   | 0.101 ¥ |
| **Ki67, %**                 |               |               |         |
| Minimum                     | 1             | 2             | -       |
| Maximum                     | 18            | 18            | -       |

Φ - Fisher’s exact test; SD – Standard deviation; ¥ - Two sample t-test; £ - Wilcoxon rank-sum (Mann-Whitney) test; TILs - Tumor Infiltrating Lymphocytes; ¶ - Two-sided Chi-squared.
|                          | OSNA negative N = 16 | OSNA positive N = 16 | \( p \)-value |
|--------------------------|----------------------|----------------------|----------------|
| **Mean ± SD**            | 7.3 ± 5.1            | 10.4 ± 4.0           | \( 0.034 \)^¥ |
| **TILs, %**              |                      |                      |                |
| Low TILs (%)             | 37.5% (n = 6)        | 31.3% (n = 5)        | \( 0.878 \)^Φ |
| Intermediate TILs (%)    | 31.3% (n = 5)        | 25.0% (n = 4)        |                |
| High TILs (%)            | 31.3% (n = 5)        | 37.5% (n = 6)        |                |
| Unknown                  | 0.0% (n = 0)         | 6.3% (n = 1)         |                |
| **Mean ± SD**            | 29.4 ± 28.6          | 32.0 ± 17.6          | \( 0.647 \)^£ |

Φ - Fisher’s exact test; SD – Standard deviation; \( ¥ \) - Two sample t-test; £ - Wilcoxon rank-sum (Mann-Whitney) test; TILs - Tumor Infiltrating Lymphocytes; ¶ - Two-sided Chi-squared.

The SLNs were identified under combined techniques and the number of removed SLNs were similar in both groups (Table 3). In OSNA positive group, the mean number of metastatic SLNs was 1.1 ± 0.3 (Table 3 and Additional file 2). Micrometastases were found in 43.8% (n = 7) and macrometastases in 56.2% (n = 9) (Table 3 and Additional file 2). In metastatic SLNs, the mean TTL was 121 238.1 ± 213 294.7. In SLNs with micrometastases the mean TTL was 1 394.3 ± 1 750.0 and in SLNs with macrometastases the mean TTL was 214 450 ± 250 915.2 (\( p < 0.001 \)). ALND was performed in 6 patients (37.5%) and 4 out of 6 patients had metastases in non-sentinel LNs (Table 3). Among patients submitted to ALND, the mean total number of metastatic LNs (sentinel and non-sentinel) was 2.7 ± 0.9 (minimum = 1; maximum = 7) and macrometastatic LNs was 2.5 ± 1.0 (minimum = 1; maximum = 7). In the group with metastases in non-sentinel LNs (n = 4) the mean TTL was 375 975 ± 292 423. Considering the small ALND sample (n = 6), there was no statistically significant correlation between the TTL and the number of non-sentinel metastatic LNs (\( r_s = 0.383; \ p = 0.454 \)). However, globally, there was a positive statistically significant correlation with a strong Spearman correlation coefficient between the TTL and the total number of LNs with metastases (sentinel and non-sentinel) (\( r_s = 0.675; \ p = 0.004 \)). Finally, there was also a positive statistically significant correlation between tumor diameter and TTL, with a very strong Spearman correlation coefficient (\( r_s = 0.870; \ p < 0.001 \)). Nevertheless, there were no statistically significant correlations between TTL and other parameters such as age, Body Mass Index (BMI), grade, ER, PR, Ki67, or TILs.
Table 3
Characteristics of the SLNs, comparing patients with OSNA negative SLNs (pN0) and patients with OSNA positive SLNs (pN1mi and pN1).

|                          | OSNA negative N = 16 | OSNA positive N = 16 | p-value |
|--------------------------|----------------------|----------------------|---------|
| **Technique for SLNs detection (%)** |                      |                      |         |
| Patent blue and radioisotope | 56.2% (n = 9)        | 75.0% (n = 12)       | 0.229 Φ |
| Superparamagnetic iron oxide | 43.8% (n = 7)        | 25.0% (n = 4)        |         |
| **Number of removed SLNs** |                      |                      |         |
| Minimum                  | 1                    | 1                    |         |
| Maximum                  | 3                    | 4                    |         |
| Mean ± SD                | 1.7 ± 0.8            | 1.8 ± 1.0            | 0.822 £ |
| **Number of metastatic SLNs** |                    |                      |         |
| 1 metastatic SLN (%)     | -                    | 93.8% (n = 15)       |         |
| 2 metastatic SLNs (%)    | -                    | 6.2% (n = 1)         |         |
| Mean ± SD                | -                    | 1.1 ± 0.3            |         |
| **OSNA result (%)**      |                      |                      |         |
| Negative (pN0)           | 100% (n = 16)        | -                    |         |
| Micrometastases (pN1mi)  | -                    | 43.8% (n = 7)        |         |
| Macrometastases (pN1)    | -                    | 56.2% (n = 9)        |         |
| **OSNA selected sample result** |                  |                      |         |
| Minimum                  | <160                 | 280                  |         |
| Maximum                  | <160                 | 730 000              |         |
| Mean ± SD                | -                    | 118 560 ± 211 763.5  |         |
| **TTL**                  |                      |                      |         |
| Minimum                  | -                    | 280                  |         |
| Maximum                  | -                    | 730 000              |         |
| Mean ± SD                | -                    | 121 238.1 ± 213 294.7|         |
| **ALND (%)**             | -                    | 37.5% (n = 6)        |         |
| If ALND:                 | -                    | 66.7% (n = 4)        |         |
| **Number of non-sentinel LNs with metastases** |                  |                      |         |
| Minimum                  | -                    | 0                    |         |
| Maximum                  | -                    | 6                    |         |

SLN(s) - Sentinel Lymph Node(s); Φ - Fisher’s exact test; SD - Standard deviation; £ - Wilcoxon rank-sum (Mann-Whitney) test; TTL - Total Tumor Load, in copies/µL of CK19 mRNA; ALND - Axillary lymph node dissection; LNs - Lymph Nodes
### 3.2 GENE EXPRESSION ANALYSIS

We obtained a successful transcript analysis in the 32 cases (100%). Data concerning RNA-seq have been deposited in NCBI's Gene Expression Omnibus (accession number GSE210006).

The result of PCA for the two principal components, including all transcripts and all samples, is shown in Fig. 1.

The results suggest a high variability and complex pattern of gene expression between samples as the two principal components only explain 41% of samples' variability. For genes heavily influencing first principal component (PC1) there is higher variation between groups (mainly between pN1 and pN1mi + pN0) than between samples in the same group (as sample-groups are differentiated along PC1). Genes heavily influencing the second principal component (PC2) mainly explain intra group-sample variability, showing a high dispersion. Considering PC1, two pN1 samples stand out, S24 and S19.

A differential gene expression analysis was performed using the DESeq2 R package to compare between sample groups (pN0, pN1mi and pN1). Comparing patients' SLNs with and without metastases (OSNA positive versus OSNA negative), 7 DEGs were identified as upregulated (1.8%) and none as downregulated (Table 4). Comparing OSNA positive with micrometastases (pN1mi) and OSNA negative SLNs (pN0), no DEGs were identified. Comparing OSNA positive with macrometastases and OSNA negative SLNs, 11 DEGs were identified as upregulated (2.8%) and none as downregulated (Table 5). Lastly, comparing OSNA positive SLNs with macrometastases and OSNA positive SLNs with micrometastases, 7 DEGs were identified as upregulated (1.8%) and none as downregulated (Table 6).
Table 4
Genes differentially expressed between OSNA positive SLNs (pN1 and pN1mi) and OSNA negative SLNs (pN0).

| Gene symbol * | Gene name * | Main function(s) ** | Fold Change | FDR p-value |
|---------------|-------------|---------------------|-------------|-------------|
| KRT7          | Keratin 7   | Tumor marker        | 315.2       | < 0.001     |
| VTCN1         | V-set domain containing T cell activation inhibitor 1 | Inhibitory immune checkpoint | 160.9 | < 0.001 |
| CD44          | CD44 molecule (Indian blood group) | Adhesion, migration; Marker of effector and memory T cells | 17.5 | < 0.001 |
| GATA3         | GATA Binding protein 3 | Transcription factor; Th2 cells and ILC2 | 6.0 | < 0.001 |
| ALOX15B       | Arachidonate 15-lipoxygenase type B | Macrophage enzyme | 5.7 | 0.040 |
| RORC          | RAR related orphan receptor C | Transcription factor; Circadian rhythm; Th17 differentiation | 3.5 | 0.025 |
| NECTIN2       | Nectin cell adhesion molecule 2 | Adhesion, migration; Modulator of T-cell signaling | 3.3 | 0.042 |

Differentially expressed genes were identified using the DESeq2 R package with adjusted \( p \)-value cut-off < 0.05 and a log2 fold change cut-off of 0.58. Log2 fold change values were converted to fold change to facilitate interpretation of data. Non-adjusted \( p \)-value was < 0.001 for all markers.

*According to HGNC (HUGO Gene Nomenclature Committee).

**Main function(s) according to Oncomine™ Immune Response Research Assay (Thermo Fisher Scientific, USA) (Additional file 1) and GeneCards® (RRID: SCR_002773).

Th2 - T helper cells of type 2; ILC2 - Type 2 innate lymphoid cells; Th 17 - T helper cells of type 17
Table 5
Genes differentially expressed between OSNA positive SLNs with macrometastases (pN1) and OSNA negative SLNs (pN0).

| Gene symbol * | Gene name * | Main function(s) ** | Fold Change | FDR p-value |
|---------------|-------------|---------------------|-------------|-------------|
| VTCN1         | V-set domain containing T cell activation inhibitor 1 | Inhibitory immune checkpoint | 897.6       | < 0.001     |
| KRT7          | Keratin 7   | Tumor marker        | 548.7       | < 0.001     |
| CD44          | CD44 molecule (Indian blood group) | Adhesion, migration; Marker of effector and memory T cells | 123.6       | < 0.001     |
| GATA3         | GATA Binding protein 3 | Transcription factor; Th2 cells and ILC2 | 9.5         | < 0.001     |
| ALOX15B       | Arachidonate 15-lipoxygenase type B | Macrophage enzyme | 9.3         | < 0.001     |
| LRG1          | Leucine rich alpha-2-glycoprotein 1 | Signal transduction; Neutrophil degranulation | 8.3         | < 0.001     |
| RORC          | RAR related orphan receptor C | Transcription factor; circadian rhythm; Th17 differentiation | 5.2         | < 0.001     |
| NECTIN2       | Nectin cell adhesion molecule 2 | Adhesion, migration; Modulator of T-cell signaling | 4.9         | < 0.001     |
| CD276         | CD276 molecule | Inhibitory immune checkpoint | 3.4         | < 0.001     |
| FOXM1         | Forkhead box M1 | Transcription factor; Proliferation | 3.3         | 0.016       |
| IGF1R         | Insulin like growth factor 1 receptor | Growth factor receptor; Proliferation | 3.0         | 0.016       |

Differentially expressed genes were identified using the DESeq2 R package with adjusted p-value cut-off < 0.05 and a log2 fold change cut-off of 0.58. Log2 fold change values were converted to fold change to facilitate interpretation of data. Non-adjusted p-value was < 0.001 for all markers.

*According to HGNC (HUGO Gene Nomenclature Committee).

**Main function(s) according to Oncomine™ Immune Response Research Assay (Thermo Fisher Scientific, USA) (Additional file 1) and GeneCards® (RRID: SCR_002773).

Th2 - T helper cells of type 2; ILC2 - Type 2 innate lymphoid cells; Th 17 - T helper cells of type 17
Table 6
Genes differentially expressed between OSNA positive SLNs with macrometastases (pN1) and OSNA positive SLNs with micrometastases (pN1mi).

| Gene symbol * | Gene name * | Main function(s) ** | Fold Change | FDR p-value |
|---------------|-------------|----------------------|-------------|-------------|
| CD44          | CD44 molecule (Indian blood group) | Adhesion, migration; Marker of effector and memory T cells | 77.2 | < 0.001 |
| KRT7          | Keratin 7   | Tumor marker         | 31.8        | 0.012       |
| ALOX15B       | Arachidonate 15-lipoxygenase type B | Macrophage enzyme | 8.8 | 0.012 |
| GATA3         | GATA Binding protein 3 | Transcription factor; Th2 cells and ILC2 | 6.8 | < 0.001 |
| LRG1          | Leucine rich alpha-2-glycoprotein 1 | Signal transduction; Neutrophil degranulation | 6.2 | 0.024 |
| RORC          | RAR related orphan receptor C | Transcription factor; circadian rhythm; Th17 differentiation | 4.1 | 0.012 |
| NECTIN2       | Nectin cell adhesion molecule 2 | Adhesion, migration; Modulator of T-cell signaling | 3.8 | 0.029 |

Differentially expressed genes were identified using the DESeq2 R package with adjusted p-value cut-off < 0.05 and a log2 fold change cut-off of 0.58. Log2 fold change values were converted to fold change to facilitate interpretation of data. Non-adjusted p-value was < 0.001 for all markers.

*According to HGNC (HUGO Gene Nomenclature Committee).

**Main function(s) according to Oncomine™ Immune Response Research Assay (Thermo Fisher Scientific, USA) (Additional file 1) and GeneCards® (RRID: SCR_002773).

Th2 - T helper cells of type 2; ILC2 - Type 2 innate lymphoid cells; Th 17 - T helper cells of type 17

To allow an overview of the levels of expression of the 11 genes identified as being differential expressed between sample groups (pN0, pN1mi and pN1), these values are described in Table 7. A gradient of expression from pN0 to pN1 is evidenced, with some genes showing very low levels of expression in no metastatic SLNs (pN0).
Table 7
Normalized expression levels of the 11 genes identified as differentially expressed, comparing pN0, pN1mi and pN1.

| Gene   | pN0  | pN1mi | pN1  |
|--------|------|-------|------|
|        | N = 16 | N = 7   | N = 9 |
| KRT7   | Minimum | 0.0     | 69.5   |
|        | Maximum | 43.5   | 20 760.6 |
|        | Mean ± SD | 8.3 ± 12.2 | 4 519.0 ± 7 006.0 |
| VTCN1  | Minimum | 0.0     | 1.2    |
|        | Maximum | 0.0     | 1 050.5 |
|        | Mean ± SD | 0.0 ± 0.0 | 158.7 ± 343.8 |
| CD44   | Minimum | 0.0     | 8.3    |
|        | Maximum | 14.8    | 6 361.5 |
|        | Mean ± SD | 7.4 ± 4.1 | 929.0 ± 2 050.4 |
| GATA3  | Minimum | 280.8   | 666.2  |
|        | Maximum | 934.9   | 17 988.7 |
|        | Mean ± SD | 560.4 ± 190.6 | 5 338.1 ± 5 460.9 |
| ALOX15B| Minimum | 0.8     | 5.8    |
|        | Maximum | 43.4    | 461.5  |
|        | Mean ± SD | 11.6 ± 10.5 | 106.9 ± 144.9 |
| RORC   | Minimum | 78.2    | 147.4  |
|        | Maximum | 141.9   | 1 862.0 |
|        | Mean ± SD | 107.5 ± 17.7 | 554.4 ± 591.3 |
| NECTIN2| Minimum | 48.7    | 136.3  |
|        | Maximum | 231.2   | 2 049.8 |
|        | Mean ± SD | 131.3 ± 51.7 | 641.4 ± 617.0 |
| LRG1   | Minimum | 20.6    | 61.2   |
|        | Maximum | 245.7   | 2 210.4 |

SD – Standard deviation
|        | pN0  | pN1mi | pN1   |
|--------|------|-------|-------|
| pN0    |      |       |       |
| N = 16 |      |       |       |
| Mean ± SD | 70.3 ± 54.5 | 95.0 ± 41.4 | 586.9 ± 765.4 |
| CD276  |      |       |       |
| Minimum | 35.6 | 46.5  | 48.7  |
| Maximum | 127.7 | 155.2 | 875.7 |
| Mean ± SD | 72.0 ± 28.2 | 92.1 ± 36.6 | 247.6 ± 260.1 |
| FOXM1  |      |       |       |
| Minimum | 52.5 | 61.7  | 107.5 |
| Maximum | 182.5 | 230.7 | 1463.6 |
| Mean ± SD | 112.2 ± 35.9 | 110.7 ± 56.2 | 367.5 ± 423.1 |
| IGF1R  |      |       |       |
| Minimum | 645.2 | 823.8 | 794.1 |
| Maximum | 2090.1 | 1777.4 | 11664.7 |
| Mean ± SD | 1241.1 ± 382.5 | 1237.7 ± 290.7 | 3768.6 ± 3344.0 |

SD – Standard deviation

3.3. Statistical analysis between DEGs and relevant clinicopathologic parameters

The results of Spearman correlation between relevant clinicopathologic parameters and the normalized expression levels of the 11 identified DEGs are shown in Table 8. This analysis concerns all samples (pN0, pN1mi and pN1) except for OSNA sample result and for TTL. For these parameters, only patients with OSNA positive samples were included: the result in OSNA negative samples did not allow Spearman correlation and TTL, by definition, was the sum of the total number of CK19 mRNA copies only in positive SLNs. (15, 18)
As shown in Table 8, Spearman correlation demonstrated a statistically significant positive correlation between TTL and the normalized expression levels of the majority of the DEGs (KRT7, VTCN1, CD44, GATA3, ALOX15B, RORC, NECTIN2, LRG1, CD276, FOXM1, and IGF1R). The OSNA sample result (number of copies of CK19 mRNA/µl) of the analyzed OSNA positive samples also showed statistically significant positive correlations with the same DEGs except for LRG1. There were also statistically
significant positive correlations between the tumor diameter and the normalized expression levels of \textit{KRT7}, \textit{GATA3} and \textit{FOXM1}; between the tumor grade and the normalized expression levels of \textit{KRT7}, \textit{VTCN1}, \textit{CD44}, \textit{GATA3}, \textit{RORC}, \textit{NECTIN2}, \textit{LRG1}, \textit{CD276} and \textit{FOXM1}; and between the PR and the normalized expression levels of \textit{ALOX15B} and \textit{NECTIN2}. There were no statistically significant correlations between the normalized expression levels of any of the 11 identified DEGs and age, BMI, Ki67, ER or TILs.

Lastly, for the subsequent multiple regression analysis (with each DEG as dependent variable and the respective significant clinicopathologic parameters as independent variables), as TTL and OSNA sample result were almost coincident ($r_s = 0.997; p < 0.001$) (Additional file 2) and TTL have a greater and proven clinical relevance, only TTL was considered.\textsuperscript{(13, 15, 19)} For \textit{KRT7}, \textit{VTCN1}, \textit{CD44}, \textit{GATA3}, \textit{ALOX15B} and \textit{RORC}, a multiple linear regression with normalized expression levels of each DEG as dependent variable and significant clinicopathologic parameters (Table 8) as independent variables was attempted, but none of the independent variables were statistically significant. However, multiple linear regression with normalized expression levels of \textit{NECTIN2}, \textit{LRG1}, \textit{CD276}, \textit{FOXM1} and \textit{IGF1R} as dependent variables and the corresponding significant clinicopathologic parameters (Table 8) as independent variables revealed: an independent linear relationship between TTL and normalized expression levels of \textit{NECTIN2} ($b = 0.002; p < 0.001; R^2 = 0.791$); an independent linear relationship between TTL and normalized expression levels of \textit{LRG1} ($b = 0.002; p = 0.004; R^2 = 0.578$); an independent linear relationship between TTL and normalized expression levels of \textit{CD276} ($b = 0.001; p = 0.003; R^2 = 0.652$); an independent linear relationship between TTL and normalized expression levels of \textit{FOXM1} ($b = 0.001; p = 0.003; R^2 = 0.822$); and an independent linear relationship between TTL and normalized expression levels of \textit{IGF1R} ($b = 0.012; p < 0.001; R^2 = 0.830$).

### 3.4. Clusters

To identify patterns of gene expression within the 32 samples, a hierarchical clustering heatmap was constructed using the 11 identified DEGs (Fig. 2). Three main clusters were identified: cluster 1 including two cases with macrometastases (pN1), cluster 2 with most pN1 cases but also with two micrometastases cases (pN1mi) and cluster 3 aggregating N0 and most pN1mi cases (Fig. 2; Table 9).
Table 9
Comparison of the main characteristics between the three identified clusters.

|                      | Cluster 1 (N = 2) | Cluster 2 (N = 8) | Cluster 3 (N = 22) | p-value |
|----------------------|-------------------|-------------------|-------------------|---------|
| **Age, years (mean ± SD)** | 53.0 ± 7.1        | 55.9 ± 7.9        | 59.6 ± 8.0        | 0.344 Δ |
| **BMI, kg/m2 (mean ± SD)** | 28.6 ± 6.1        | 26.6 ± 2.4        | 25.9 ± 4.4        | 0.645 Δ |
| **Tumor diameter, mm**  |                  |                   |                   |         |
| Minimum              | 16                | 12                | 2                 |         |
| Maximum              | 35                | 29                | 25                |         |
| Mean ± SD            | 25.5 ± 13 - 4¥    | 18.8 ± 5.5        | 13.2 ± 5.7        | 0.009 Δ |
| **Tumor grade**      |                  |                   |                   |         |
| Minimum              | 2                 | 1                 | 1                 |         |
| Maximum              | 3                 | 3                 | 2                 |         |
| Mean ± SD            | 2.5 ± 0.7£        | 1.9 ± 0.6         | 1.3 ± 0.5£        | 0.010 Ψ |
| **ER, % (mean ± SD)** | 100.0 ± 0.0       | 94.4 ± 7.3        | 93.2 ± 8.4        | 0.435 Ψ |
| **PR, % (mean ± SD)** | 95.0 ± 7.1        | 63.8 ± 23.0       | 69.1 ± 25.4       | 0.284 Δ |
| **Ki67, % (mean ± SD)** | 8.5 ± 4.9        | 10.5 ± 4.1        | 8.3 ± 5.1         | 0.540 Δ |
| **Lymphovascular invasion (%)** | 0.0% (n = 0)   | 50.0% (n = 4)     | 18.2% (n = 4)     | 0.144 Φ |
| **TILs, % (mean ± SD)** | 20.0 ± 14.1       | 43.1 ± 26.3       | 26.9 ± 27.4       | 0.232 Ψ |
| **All SLNs pN0 (%)**  | 0.0% (n = 0)      | 0.0% (n = 0)      | 72.7% (n = 16)    | 0.001 Φ |
| **OSNA sample result** |                  |                   |                   |         |
| Minimum              | 12 000            | 3 300             | <160              |         |
| Maximum              | 730 000           | 430 000           | 8 200             |         |
| Mean ± SD            | 371 000 ± 507 702.7 | 143 100 ± 169 295.7 | -                 |         |
| **Number of metastatic SLNs** |                  |                   |                   |         |
| Minimum              | 1                 | 1                 | 0                 |         |
| Maximum              | 1                 | 2                 | 1                 |         |
| Mean ± SD            | 1.0 ± 0.0         | 1.1 ± 0.4£        | 0.3 ± 0.5£        | < 0.001 Ψ |
| **TTL**              |                  |                   |                   |         |
| Minimum              | 21 900            | 3 300             | 280               |         |
| Maximum              | 730 000           | 430 000           | 8 200             |         |

SD – Standard deviation; Δ - One-way ANOVA; BMI - Body Mass Index; * p < 0.05; ¥ - Two sample t-test (between cluster 1 versus cluster 2 + 3; cluster 2 versus cluster 1 + 3; cluster 3 versus cluster 1 + 2, respectively); ** p < 0.001; £ - Wilcoxon rank-sum (Mann-Whitney) test (between cluster 1 versus cluster 2 + 3; cluster 2 versus cluster 1 + 3; cluster 3 versus cluster 1 + 2, respectively); Ψ - Kruskal-Wallis test; Φ - Fisher's exact test; TTL - Total Tumor Load, in copies/µL of CK19 mRNA.
The two cases with macrometastases of cluster 1 were from patients that also had non-sentinel LNs with macrometastases and vessels embolization on the histologic examination of the non-sentinel LNs. These samples were the two pN1 outliers previously identified in the PCA analysis (Fig. 1). The mean TTL of these two cases was high (375 950.0 ± 500 702.3) but some cases in cluster 2 showed higher values (Table 9 and Additional file 2). In cluster 1, the mean number of total LNs (SLNs and non-sentinel LNs) with macrometastases was also significantly higher (5.0 ± 2.8 versus 0.3 ± 0.5 in cluster 2 + cluster 3; p < 0.001). Globally, all the 11 DEGs had higher expression in cluster 1 (Table 10).
Table 10
Comparison of gene expression levels of DEGs between the three identified clusters.

| Cluster 1  | Cluster 2  | Cluster 3  | p-value |
|-----------|-----------|-----------|---------|
| (N = 2)   | (N = 8)   | (N = 22)  |         |
| KRT7 (mean ± SD) | 16 060.7 ± 6646.6*£ | 1182.2 ± 901.6**£ | 10.1 ± 16.9**£ | < 0.001 Ψ |
| VTCN1 (mean ± SD) | 544.7 ± 715.2*£ | 51.2 ± 85.7**£ | 0.3 ± 0.7**£ | < 0.001 Ψ |
| CD44 (mean ± SD) | 3347.6 ± 4262.3*£ | 212.6 ± 256.3**£ | 7.6 ± 4.3**£ | < 0.001 Ψ |
| GATA3 (mean ± SD) | 13 848.2 ± 5855.6*£ | 2811.1 ± 1592.4**£ | 559.6 ± 171.6**£ | < 0.001 Ψ |
| ALOX15B (mean ± SD) | 270.5 ± 270.2*£ | 52.8 ± 64.5 | 12.2 ± 10.3*£ | 0.022 Ψ |
| RORC (mean ± SD) | 1540.1 ± 455.2*£ | 255.3 ± 127.2*£ | 115.6 ± 22.7**£ | < 0.001 Ψ |
| NECTIN2 (mean ± SD) | 1185.1 ± 1222.8 | 457.9 ± 343.7*£ | 137.3 ± 49.0**£ | < 0.001 Ψ |
| LRG1 (mean ± SD) | 1384.6 ± 1167.9*£ | 338.2 ± 493.2*£ | 137.3 ± 49.0**£ | < 0.001 Ψ |
| CD276 (mean ± SD) | 633.5 ± 342.5*£ | 139.8 ± 71.0*£ | 74.5 ± 31.8**£ | < 0.001 Ψ |
| FOXM1 (mean ± SD) | 819.1 ± 911.5 | 222.1 ± 119.4*£ | 111.9 ± 38.3*£ | 0.009 Ψ |
| IGF1R (mean ± SD) | 7452.8 ± 5956.5*£ | 2555.5 ± 1703.5*£ | 1231.3 ± 369.5*£ | 0.006 Ψ |

SD – Standard deviation; * p < 0.05; £ - Wilcoxon rank-sum (Mann-Whitney) test (between cluster 1 versus cluster 2 + 3; cluster 2 versus cluster 1 + 3; cluster 3 versus cluster 1 + 2, respectively); ** p < 0.001; Ψ - Kruskal-Wallis test.

Cluster 2 included only OSNA positive cases: 6 with macrometastases and 2 with micrometastases. The mean TTL was 147 218.8 ± 173 519.8 (Table 9). Importantly, the two cases of SLNs with micrometastases that clustered with this group had a high TTL (3300 and 4500, respectively) (Additional file 2), close to the established cut-off of macrometastases (5 000). Globally, the 11 DEGs were less expressed than in cluster 1 but more expressed than in cluster 3 (Table 10).

On the other hand, the predominantly OSNA negative cluster (cluster 3) included 16 pN0 patients, 5 of the 7 pN1mi patients and one pN1 patient. The mean TTL of the six OSNA positive patients in this cluster 3 was low (1 693 ± 3 189.9). The patients with micrometastases had low TTL (minimum = 280; maximum = 620) and the only patient pN1 (with macrometastases) in this cluster had the lowest TTL among the patients with macrometastases (8 200) (Additional file 2). Cluster 3 had the lowest gene expressions of this 11 DEGs (Table 10). Moreover, considering only the patients with metastatic SLNs (n = 16), in cluster 3 (n = 6) the gene expression levels of the DEGs were globally significantly lower when compared to gene expression levels of clusters 1 and 2 (Additional file 3).

Finally, besides tumor diameter and tumor grade, the comparison of clinical and other tumor pathological characteristics (hormone receptors, LVI, Ki67 and TILs) between the three clusters did not reveal any statistically significant difference (Table 9).

4. Discussion

The cross-talk between immune cells and tumor cells modulates tumor metastases and response to therapy.(45) By binding to inhibitory receptors on immune cells, metastatic cancer cells can disrupt tumor immunity and establish a pro-tumoral microenvironment.(45) Tumors escape immune-mediated recognition through multiple mechanisms.(46) During chronic tumor antigen exposure, T cells become dysfunctional/exhausted and upregulate various checkpoint inhibitory receptors that limit T cell survival and function.(46) In physiological conditions, immune checkpoints (as the identified DEGs VTCN1 and CD276) are crucial to prevent exaggerated inflammation, which would otherwise cause damage to the tissues; however, through upregulation of
immune checkpoints, BC cells can also acquire the ability to suppress the immune response and evade recognition and consequent elimination by the immune system.\(^{(47)}\)

Emerging literature is revealing the potential for the assessment of immune microenvironment in SLNs as predictive biomarkers for treatment by immune checkpoint inhibitors immunotherapies.\(^{(48)}\) Indeed, evaluating the immune response within the SLNs could become an easier and more informative measure of therapy efficacy than the assessment of TILs within the primary TME.\(^{(48)}\) Previous studies suggested that the presence of primary and metastatic disease promote immune suppression within the SLNs and this may need to be overcome to observe a response to immunotherapy.\(^{(48)}\)

Gene expression analysis of RNA from SLNs can be used to characterize both cancer and immune cells.\(^{(49)}\) Using a target RNA-seq, this study revealed DEGs that may be predictive biomarkers in the immune-oncology interface, focusing on the interaction of tumor cells with the microenvironment. When comparing OSNA positive with OSNA negative SLNs, 7 upregulated DEGs were identified, a number that increased to 11, when considering only macrometastatic SLNs (Tables 4 and 5). The higher number is probably related to the increased levels of expression, and consequently strengthening statistical power. The upregulated DEGs (\textit{VTCN1, KRT7, CD44, GATA3, ALOX15B, LRG1, RORC, NECTIN2, CD276, FOXM1} and \textit{IGF1R}) (Table 5), include genes mainly expressed in BC cells and genes expressed both in BC and microenvironment cells [according to GeneCards® (RRID: SCR_002773)]. Proteins encoded by these genes have different functions yet some of these functions overlap, such as proliferation (\textit{FOXM1} and \textit{IGF1R}), cell-adhesion (\textit{CD44} and \textit{NECTIN2}) and inhibition of immune response (\textit{VTCN1} and \textit{CD276}), as described in Tables 4 to 6. Remarkably, in our study, genes with an expression that could be mainly attributed to microenvironment cells, as granzymes (\textit{GZMA, GZMB, GZMH, GZMK}), \textit{CD3} (\textit{CD3D, CD3E, CD3G}), other immune system-response related genes codifying interleukins, IFN-\(\gamma\), T cell receptors (TCRs) or immune checkpoint molecules such as Programmed Cell Death-1 (PD-1) or Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4), did not show differential expression between pN0, pN1mi and pN1 samples. This lack of evidence of the involvement of other genes associated with immune system activation may possibly be related to the increased expression of the inhibitory immune checkpoints \textit{VTCN1} and \textit{CD276}.

Analysis of the expression levels of the 11 DEGs within the three groups of samples (pN0, pN1mi and pN1), as shown in Table 7, highlights the correlation with the metastatic load of SLN. Some genes, such as \textit{KRT7}, \textit{VTCN1}, \textit{CD44} or \textit{ALOX15B} had very low or even no expression in pN0 samples (Table 7), which is in accordance with the low levels of the respective proteins in LNs, previously described in literature.\(^{(50)}\) On the other hand, concerning metastatic SLNs, increased levels of CK19 mRNA copies, and, consequently, increased levels of TTL, were correlated with higher expression levels of the majority of the DEGs (\textit{KRT7}, \textit{VTCN1}, \textit{CD44, GATA3, RORC, NECTIN2, LRG1, CD276, FOXM1} and \textit{IGF1R}) (Table 8), strengthening that the changes in the LNs microenvironment associated with metastases reflect a progressive process. Yet, when evaluating gene expression in OSNA positive samples, the levels of expression cannot be assigned to specific cell types. So, for the identified DEGs, the increased levels of gene expression in the SLNs with higher copies of mRNA CK19/\(\mu l\) may be explained just by tumor load or by the simultaneous overexpression in microenvironment cells in response to metastases. Immunohistochemical studies targeting proteins codified by DEGs would clarify which cells are involved. However, independently of the cell type expressing these biomarkers, they may be useful as prognostic markers and for targeted therapies selection.

Tumor diameter, tumor grade, PR, OSNA sample result and TTL showed positive correlations with DEGs expression levels in SLNs (Table 8). For tumor diameter and tumor grade, the most probable explanation for these correlations is the association of higher values of these parameters with LN metastases. The weak but statistically significant positive correlations between PR and expression levels of \textit{ALOX15B} and \textit{NECTIN2} in SLNs hadn't been previously described in literature. Regarding TTL values and OSNA sample results, as values were almost coincident (Additional file 2), we cannot state if these correlations will stand in patients with a higher number of positive SLNs.

Furthermore, in this study, cluster analysis of samples based on the expression profile of the referred 11 DEGs established three different clusters: cluster 1 had the highest gene expression levels whereas cluster 3 had the lowest one, even when only the metastatic SLNs were considered for analysis (Table 10 and Additional file 3). The different clusters, not entirely coincident with pN0, pN1mi and pN1 classification, may relate to distinct prognosis behavior. Based on clinical and pathological characteristics, cluster 1 would have the worst prognosis and cluster 3 the best.
This study is the first in human BC patients analyzing the immune-related DEGs in the whole SLN, comparing the global microenvironment of non-metastatic and metastatic SLNs, and subdividing in micrometastases and macrometastases. A previous study compared the DEGs between metastatic and non-metastatic LN, using a microarray-based dataset (GSE4408) to evaluate 16 metastatic and 3 non-metastatic LNs of mice bearing orthotopic human BC xenografts. (51, 52) Rizwan et al., mainly focused on changes in collagen density, investigating extracellular matrix molecules and detected 13 DEGs. (51) Valente et al. compared the DEGs between metastatic and non-metastatic LNs in human BC patients (including all subtypes), however, in metastatic LNs, it was analyzed exclusively the uninvolved (“normal”) residual portion of an otherwise involved LN (using laser microdissection to collect exclusively cell populations of the LN, avoiding the bulk of the tumor and the tumor/LN margins). (53) The authors used a microarray-based dataset (HG U133A 2.0) to analyze the gene expression and concluded that immune gene expression profiles in uninvolved residual portion of metastatic LNs are significantly different from negative LNs, with 22 DEGs. (53) Blackburn et al., assessing DEGs between non-metastatic LNs in patients with other positive LNs versus non-metastatic LNs in patients with all negative LNs, did not found any DEGs, suggesting that the presence of metastatic cells within the lymphatic system does not elicit widespread changes in gene expression through the remaining LNs; rather, LNs independently respond to disseminated tumor cells. (54) Also, Rye et al., in a study using single-cell immune profiling of LNs with and without metastatic cells revealed that immune suppression occurred at early stages of local spread of BC; however, a certain tumor burden must be reached before changes in immune cell distribution can be detected. (55) Thus, the physical presence of metastatic tumor cells may be crucial to elicit a pro-metastatic niche in the LNs and, consequently, the changes in the microenvironment associated with metastases reflect alterations associated with tumor growth and progression. (52–55) Indeed, in our study, we verify that there are no DEGs between non-metastatic SLN (pN0) and SLN with micrometastases (pN1mi). However, 11 DEGs were identified as upregulated in macrometastatic SLNs (Table 5).

In our study, we used a target RNA-seq to study the transcriptomic patterns of immune response at the level of SLNs. Because RNA-seq does not rely on a predesigned complement sequence detection probe, there are no limitations such as cross-hybridization with extremely similar sequences. (56, 57) A recent study also revealed that the information provided by microarray and RNA-seq data is not completely the same; however, the transcript abundance assayed by RNA-seq provides a higher quality estimate of protein abundance, because the signal-to-noise is improved, compared to data obtained using a microarray. (58) RNA-seq is a very sensitive and specific method, as evidenced by the detection of differential expression of transcripts with very low expression, such as VTCN1, supporting the reliability of our results. Moreover, when compared to the whole transcriptome, beyond reduced costs, targeted RNA-seq protocols are optimized for the selected transcripts, showing increased sensitivity.

Considerable evidence suggests that BC metastases arise from cells undergoing epithelial-mesenchymal transition and cancer stem-like cells. A previous study using single-cell RNA-seq in BC cell lines revealed that migratory BC cells exhibited overall signatures of epithelial-mesenchymal transition and cancer stem-like cells with variable expression of marker genes, and they retained expression profiles of epithelial-mesenchymal transition over time. (59) Indeed, we verify that CD44 (a molecular marker for cancer stem cells) is overexpressed in metastatic Luminal A BC SLNs, being also a DEG between the macrometastatic and micrometastatic SLNs, suggesting that metastatic Luminal A BC cells retain expression profiles of epithelial-mesenchymal transition (Tables 4 to 7).

The immune response varies widely in metastases across different BC molecular subtypes. (45) In Luminal BC, Núñez et al., observed that Treg frequencies increased with nodal invasion, with a common transcriptomic signature shared by Tregs from tumors and nodes, including CD80 (an immune checkpoint molecule), which is significantly associated with poor patient survival. (60) In our study, by analyzing a broad spectrum of Luminal A BC SLNs (non-metastatic, micrometastatic and macrometastatic, with a wide range of TTL), transcriptomic patterns were revealed, capturing information on the molecular mechanisms and changes in immune composition. Since Luminal A BC patients with metastases in SLNs typically have a higher risk of disease progression and development of distant metastases, these SLNs’ transcriptomic patterns may translate into new therapeutic strategies, including the successful implementation of targeted immunotherapy in Luminal A BC patients.

Although BC was previously considered as a poorly immunogenic cancer, some patients with BC are now expected to benefit from selected immunotherapies. (61) However, the underlying mechanisms of immunotherapy in BC remains incompletely understood and effective clinical biomarkers for BC are still lacking. (61) The identification and optimization of a multiple-biomarker profiling for immunotherapy could help to properly select patients for treatment and to identify rational combination therapies. (49, 61)
Furthermore, biomarkers may help define the mechanism of action for different agents and help in dose selection and sequencing of drug combinations.\textsuperscript{(49)} To maximize the clinical treatment benefit of cancer immunotherapy, the prediction of the actual immune response by the identification and application of clinically useful biomarkers (therapeutic targets) is required.\textsuperscript{(47)} Indeed, an ideal BC therapeutic target needs to have a crucial role on the biology and/or survival of BC cells, to be highly expressed in BC cells (primary and metastatic) with low heterogeneity and to be expressed in differentiated BC cells as well as in BC initiating, because BC cells with low targeted biomarker expression levels tend to generate escape variants under selective pressure.\textsuperscript{(62, 63)} Furthermore, the therapeutic target expression on normal tissues must be restricted, preferably at levels below the ones required for effector mechanism activation, in order to minimize toxicity.\textsuperscript{(62, 63)}

**Strengths and limitations**

As far as we know, this is the first study in human BC patients that intended to analyze the immune-related DEGs in the whole SLN, comparing the global microenvironment of non-metastatic and metastatic SLNs. Furthermore, the metastatic SLNs were classified as micrometastatic and macrometastatic and the microenvironment was compared according to the tumor load.

To the best of our knowledge, this is also the first study in BC patients using a target RNA-Seq potentially useful for clinical translation. Previous gene expression studies in LNs used mainly microarray-based datasets. In our study, instead of microarrays, we used targeted RNA-seq, a more sensitive and specific method, with a higher quality estimate of protein abundance.

It is known that distinct transcriptomic profiles across molecular subtypes is associated with inter-tumoral heterogeneity of BC.\textsuperscript{(45)} In this study, selecting a homogeneous cohort of patients with Luminal A early BC, we established a differential immune transcriptomic profile of Luminal A BC metastatic SLNs and we were able to define three different clusters. Moreover, as the aggressive behavior of BC seems to derive from LNs metastases, these findings could help to take a further step in defining a more precise prognosis of Luminal A BC patients and improving methods of personalized treatments towards higher effectiveness and less side effects. To the best of our knowledge, this study is the first to delineate the immune transcriptomic profile of Luminal A BC SLNs metastases.

Finally, as another major strength, this study is the first to use the OSNA lysate spared sample in the search for prognosis and treatment related markers associated with tumor-microenvironment interplay and not only tumor markers. Hence, this study, using a targeted RNA-seq in the OSNA lysate spared samples, evidenced that a selected SLN gene expression profile can identify molecular markers useful as a new prediction tool for prognosis evaluation and treatment selection. The RNA extraction from OSNA lysate spared sample is easier and allows a higher RNA concentration, with higher quality when compared with formalin-fixed paraffin-embedded tumor samples and may constitute an alternative to tumor RNA characterization, particularly when the primary tumor size is small, as in the majority of current patients. This approach also has the additional advantage of maintaining the integrity of the primary tumor samples for eventually necessary future studies. Furthermore, since, by law, OSNA lysates samples are, currently, not required to be preserved, OSNA lysates would otherwise be wasted. Therefore, OSNA lysate samples have less ethical and legal implications and, nowadays, have no other utilities besides SLNs staging. Additionally, accordingly to previous DEGs studies, there is a transcriptomic similarity between primary BC and its corresponding LN metastases.\textsuperscript{(52)} Thus, this similarity may translate into new prognosis data and therapeutic strategies using the OSNA lysate spared sample from each BC patient. Lastly, as OSNA is being adopted worldwide by an increasing number of centers in other type of cancers besides BC, RNA-seq in the OSNA lysate spared samples could have a wider utility.

On the other hand, as a limitation, the OSNA lysate samples are obtained from homogenized SLNs, and thus, this approach performs a global evaluation of SLNs’ microenvironment, including tumoral and non-tumor cells. This limitation is inherent to OSNA sample. RNA-seq deconvolution analysis, a computational method that can simultaneously estimate both sample-specific cell-type proportions and cell-type-specific gene expression profiles using bulk tissue samples, is not feasible in this study because it would require a significantly higher number of targets.\textsuperscript{(64)} However, as already discussed, regardless of the cell of origin, the DEGs can be useful biomarkers.

The overexpression of several potential targets for immunotherapy in metastatic Luminal A BC SLNs represents a promising therapeutic target. However, as this was a RNA-seq study, successful targeting would require further knowledge about the amount and distribution of protein expression, because the RNA-protein correlation may be distorted by posttranscriptional regulation.
Finally, this study had a small sample size and no follow-up data. A larger cohort of patients with subsequent long-term follow-up will be necessary to enrich these results, especially regarding clusters implications.

5. Conclusions

Using a targeted RNA-seq, in OSNA lysate of SLNs from Luminal A BC patients, it was found that, in metastatic SLNs, there were upregulated immune-related genes. Globally, in metastatic SLNs, KRT7, VTCN1, CD44, GATA3, ALOX15B, RORC and NECTIN2 were upregulated. In macrometastatic SLNs, LRG1, CD276, FOXM1 and IGF1R were also upregulated. In metastatic SLNs, higher metastatic load and higher levels of TTL were correlated with higher expression levels of the majority of the DEGs. Hierarchical clustering analysis revealed three different clusters, not coincident with pN0, pN1mi and pN1 classification, suggesting that the expression profile of these genes may bring further information on current SLN evaluation.

The 11 identified DEGs codify proteins mainly involved in cancer aggressiveness and with impact in immune response. Some of the found DEGs are biomarkers potentially useful to take a further step in improving personalized treatment strategies towards higher effectiveness and less side effects.

As OSNA is being adopted worldwide in another cancers besides BC, RNA-seq in the OSNA lysate could also have utility for other cancer types. In the future, the SLN’s gene-signature study could be used in order to define a more precise prognosis and choose the best therapy according to patient characteristics, as the complex interaction between cancer and the host immune system will be the main strategic key to future personalized treatment tools.

List Of Abbreviations
| Abbreviation | Description |
|--------------|-------------|
| ALND         | Axillary lymph node dissection |
| ALOX15B      | Arachidonate 15-lipoxygenase type B |
| BC           | Breast cancer |
| BMI          | Body Mass Index |
| CHUC         | Coimbra Hospital and Universitary Centre |
| CK19         | Cytokeratin 19 |
| CTLA4        | Cytotoxic T-lymphocyte associated protein 4 |
| DCs          | Dendritic cells |
| DEG(s)       | Differentially expressed gene(s) |
| DNA          | Deoxyribonucleic acid |
| ER           | Estrogen receptor |
| FDR          | False discovery rate |
| FOXM1        | Forkhead box M1 |
| FOXP3        | Forkhead box P3 |
| GATA3        | GATA binding protein 3 |
| HBB          | Hemoglobin beta chain |
| HER2         | Human epidermal growth factor receptor 2 |
| IFN-γ        | Interferon γ |
| IGF1R        | Insulin like growth factor 1 receptor |
| ILC2(s)      | Type 2 innate lymphoid cell(s) |
| ITC(s)       | Isolated Tumor Cell(s) |
| KRT7         | Keratin 7 |
| LNs          | Lymph nodes |
| LRG1         | Leucine rich alpha-2-glycoprotein 1 |
| LVI          | Lymphovascular invasion |
| M            | Distant metastasis |
| mRNA         | Messenger ribonucleic acid |
| N            | Metastasis to the regional lymph nodes |
| NECTIN2      | Nectin cell adhesion molecule 2 |
| NGS          | Next generation sequencing |
| NST          | No special type |
| OSNA         | One step nucleic acid amplification |
| PCA          | Principal component analysis |
| PCRs         | Polymerase chain reactions |
| PD-1         | Programmed cell death-1 |
| PR           | Progesterone receptor |
### Declarations

#### Ethics approval and consent to participate

The project was approved by the Ethics Committee of Coimbra Hospital and University Centre (CHUC) (ethics approval number: CHUC-045-20).

#### Consent for publication

Not applicable.

#### Availability of data and materials

The datasets generated from RNA-seq data and analysed during the current study are available in the NCBI's Gene Expression Omnibus repository, with the accession number GSE210006.

#### Competing interests

The authors declare that they have no competing interests.

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| Abbreviation | Definition |
|--------------|------------|
| RAR          | Retinoic acid receptor |
| RNA          | Ribonucleic acid |
| RNA-seq      | RNA sequencing |
| RORC         | RAR related orphan receptor C |
| RORs         | Retinoic acid receptor (RAR) related orphan receptors |
| Rs           | Spearman's rank correlation coefficient |
| RT-LAMP      | Reverse transcription loop mediated isothermal amplification |
| SD           | Standard deviation |
| SLN(s)       | Sentinel lymph node(s) |
| T            | Tumor size |
| TCRs         | T cell receptors |
| Th17         | T helper cells of type 17 |
| Th2          | T helper cells of type 2 |
| TILs         | Tumor infiltrating lymphocytes |
| TME          | Tumor microenvironment |
| TN           | Triple negative |
| Tregs        | Regulatory T lymphocytes |
| TTL          | Total tumor load |
| VTCN1        | V-set domain containing T cell activation inhibitor 1 |
Authors’ contributions

IG contributed to conception and design of the study, patient recruitment, statistical work, interpretation of data and writing of the draft. JMR and JM performed RNAseq assays and JMR also performed RNASeq analysis. AG performed OSNA assay. VA performed TILs analysis. FSR contributed to interpretation of data and revision of the draft. FC contributed to statistical work. HCS and MFD contributed to conception and design of the study, data analysis and interpretation, and revision of the draft, HCS being mainly responsible for RNASeq results and analysis and MFD mainly responsible for general conception and clinical data and analysis. All authors read and approved the final manuscript.

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Figure 1

First two principal components analysis with all genes and samples. Each group of samples has a specific color.
Figure 2

Heatmap and hierarchical clustering with the 11 identified differentially expressed genes.

Supplementary Files

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