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# Influence of the Molecular Subtype-Dependent Immune Microenvironment of Metastatic Axillary Lymph Nodes on Breast Cancer Patient Outcomes

Carlos López[a, b] Laia Reverté[a] Ramon Bosch Príncep[a, c] Esther Sauras[a, b] Noèlia Gallardo-Borràs[a, b] Alba Fischer-Carles[a, d] Anna Korzynska[e] Marcial García-Rojo[f] Gloria Bueno[g] Lukasz Roszkowiak[e] Albert Roso-Llorach[h] Andrea Gras Navarro[a] Montserrat Llobera[i] Daniel Mata[a] Laia Adalid Llansa[a, c] Salomé Martínez-González[c] Joan F. Garcia-Fontgivell[c] Meritxell Arenas[j] Junior Gómez[j] José Antonio Izuel[k] Jordi Baucells[l] Marylène Lejeune[a, b]

[a]Pathology Department, Oncological Pathology and Bioinformatics Research Group, Hospital de Tortosa Verge de la Cinta, ICS, Institut d'Investigació Sanitària Pere Virgili (IISPV), Tarragona, Spain; [b]Department of Basic Medical Sciences, Universitat Rovira i Virgili (URV), Tarragona, Spain; [c]Pathology Department, Hospital Universitari Joan XXIII, Tarragona, Spain; [d]BCN MedTech, Department of Engineering, Universitat Pompeu Fabra, Barcelona, Spain; [e]Laboratory of Processing and Analysis of Microscopic Images, Nałęcz Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences (IBIB PAN), Warsaw, Poland; [f]Pathology Department, Hospital Universitario Puerta del Mar, Cadiz, Spain; [g]VISILAB, Universidad de Castilla-La Mancha, Ciudad Real, Spain; [h]Institut Universitari d'Investigació en Atenció Primària Jordi Gol (IDIAP Jordi Gol), Barcelona, Spain; [i]Oncology Department, Hospital de Tortosa Verge de la Cinta, ICS, IISPV, Tortosa, Spain; [j]Radiation Oncology Department, Hospital Universitari Sant Joan de Reus, IISPV, URV, Reus, Spain; [k]Radiology Department, Hospital de Tortosa Verge de la Cinta, ICS, IISPV, Tortosa, Spain; [l]Informatics Department, Hospital de Tortosa Verge de la Cinta, ICS, IISPV, Tortosa, Spain

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**Keywords**
Breast cancer subtypes · Immune profile · Metastatic axillary lymph nodes · Cancer-specific survival · Time to progression

**Abstract**
***Introduction:*** Metastasis in the axillary lymph nodes (ALNs) occurs in 30–50% of breast cancers (BCs), where residing immune cells play a crucial role in disease progression. Specifically at diagnosis, the immune elements infiltrating the primary tumour are among the best established prognostic factors. However, their prognostic value in the metastatic ALNs ($ALNs^+$) is poorly understood. ***Methods:*** We aimed to retrospectively assess the immune populations of $ALNs^+$ in luminal A (LA) and triple-negative BC (TNBC) patients

Carlos López and Laia Reverté contributed equally to this work.

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Correspondence to:
Laia Reverté, laia.revertecalvet@gmail.com
Esther Sauras, esauras.ebre.ics@gencat.cat

using immunohistochemistry, to compare it with non-metastatic ALNs ($ALNs^-$), and to determine their relationship with patient outcomes. ***Results:*** We found differences in the immune concentrations of matched ALNs ($ALNs^-$ vs. $ALNs^+$) from patients with positive nodal status in either LA or TNBC subtypes. In contrast, compared with LA, the levels of immune cells in $ALNs^-$ of the TNBC profile differ much more from $ALNs^+$ than in the LA subtype, regardless of the nodal status. In addition, TNBC patients with higher levels of CD4 and CD8 lymphocytes in $ALNs^+$ have worse cancer-specific survival (CSS) and higher levels of CD83 dendritic cells (DCs) are related to worse CSS and time to progression (TTP). Conversely, LA patients with higher levels of CD21 DC showed better TTP. ***Conclusion:*** Our results showed that ALN immune profiles and their influence on disease evolution vary by molecular BC subtype and nodal status, suggesting that accurate ALN immune profiling at diagnosis could provide new insights into the immune BC landscape. These observations require validation in larger, prospective cohorts before they can be reliably used to inform clinical decision-making.

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## Introduction

Of all cancer types, breast cancer (BC) is that most commonly diagnosed in women (31.0%) and the leading cause of cancer deaths (15.0%) [1]. BC is an inherently molecularly heterogeneous disease, with four surrogate subtypes being distinguished: luminal A (LA)-like, luminal B-like, human epidermal growth factor receptor 2-positive ($HER2^+$), and triple-negative (TN). These subtypes are associated with distinct prognoses [2]. BC does not consist solely of neoplastic cells but also involves cells of the tumour microenvironment. Of the various cell components in the tumour microenvironment, immune cells are one of the elements that can influence the tumour development and cancer progression [3, 4]. However, immune cells can play two opposing roles, depending on their cell subtype and the surrounding microenvironment. They can suppress tumour growth, or their activity can be restrained by the evolution of the tumour [5]. In this regard, tumour-infiltrating lymphocytes, which are a mixture of different lymphocyte subtypes, composed mainly of T cells, but also B and natural killer cells [6], are recognized as good prognostic factors in BC, especially in $HER2^+$ and TNBC subtypes [7, 8]. Besides BC subtypes and tumour size, axillary lymph node (ALN) status is one of the most important prognostic factors, since the ALN is one of the first sites to which BC metastasizes. Therefore, determining the ALN status at diagnosis is crucial to properly predicting cancer prognosis and survival [9]. ALNs, like all other lymph nodes in the body, are secondary lymphoid organs in which immune cells reside and where they can identify and fight infectious agents and cancer cells by generating an immune response. Most studies of ALNs have focussed on metastatic ALNs ($ALNs^+$), and immune cell activation has been reported [10]; however, the immune response in $ALNs^+$ has been mainly associated with an immunosuppressive pattern [11, 12]. Furthermore, non-metastatic ALN ($ALNs^-$) are thought to be more immunologically competent than $ALNs^+$ [11, 13], and different immune response gene expressions have been described in the $ALNs^-$ of patients with neighbour $ALNs^+$ [14]. In addition, we previously demonstrated that certain immune population levels in $ALNs^-$ are associated with the presence of $ALNs^+$ in their neighbourhood [15] and with patient outcomes [16] and that $ALNs^-$ of TNBC patients contained higher concentrations of markers related to immune tolerance compared with LA [17]. Nevertheless, despite the profound involvement of BC molecular subtypes in BC behaviour, there have so far been very few studies of ALNs that consider BC subtypes and the relevance of immune populations to patient survival and relapse. Therefore, the present study aimed to investigate the differences in immune cell concentrations in $ALNs^+$ between LA and TNBC patients, to compare these concentrations with those in $ALNs^-$ of patients with and without other metastatic ALNs (the latter having been described in our previous study [17]), and to determine the influence of $ALN^+$ immune populations together with clinical-pathological variables on cancer-specific survival (CSS) and time to progression (TTP), overall and for the separate BC subtypes.

## Materials and Methods

### *Clinical-Pathological Characteristics of Participants*

The present work was carried out on a retrospective cohort of 72 patients diagnosed with invasive BC between 1995 and 2008, of whom 46 (63.9%) were LA and 26 (36.1%) were TNBC subtypes. To be included in the study, patients had to meet two criteria: (1) the absence of distant metastases at the time of diagnosis and (2) the

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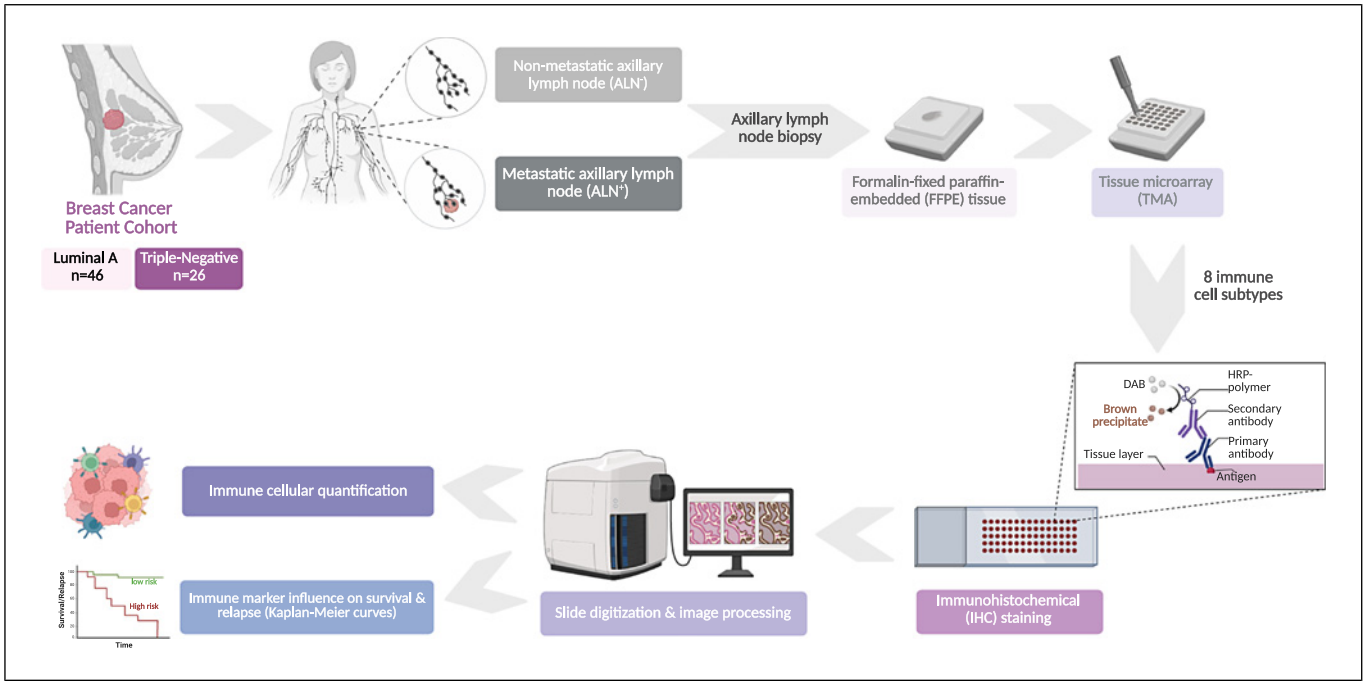



**Fig. 1.** Flowchart of the study design, sample processing, and data analysis.

availability of lymph node biopsies with enough representative tissue. The exclusion criteria were as follows: (1) insufficient tissue or (2) samples identified as sentinel ALN biopsies. The schema of the sample study is detailed in Figure 1.

We examined the biopsies of matched ALNs from patients with proven axillary metastasis at diagnosis ($ALNs^{+}$): metastatic ALNs ($ALNs^{+/+}$) and non-metastatic ALNs ($ALNs^{-/+}$). They were obtained from the Tumour Banks (Biobank IISPV) of the Hospital de Tortosa Verge de la Cinta (HTVC) and the Hospital Joan XXIII (HUJ23), Tarragona, Spain. Moreover, for comparative purposes, we used the immune data obtained in $ALNs^{-}$ of patients without nodal metastasis at diagnosis ($ALNs^{-/-}$) from our previously studied cohort of patients [16, 17]. A schematic illustration of the sample collection structure and the definition of ALNs is provided in online supplementary Figure 1 (for all online suppl. material, see https://doi.org/10.1159/000550086).

Clinical, pathological, and immune data germane to the study were compiled and are presented in tables (1–3). Patients were followed up for 10 years from the time of diagnosis or until the date of death. Of the patients included in the study, 19.6% of LA and 19.2% of TNBC patients received neoadjuvant therapy, 95.7% of LA and 96.2% of TNBC received adjuvant therapy (hormonal therapy and/or chemotherapy), and 76.1% of LA and 84.6% of TNBC received radiotherapy.

The endpoints include CSS, which was defined as the time from diagnosis to the end of follow-up or death due to BC, and TTP, defined as the time from the date of first diagnosis until the end of follow-up (non-relapsed patients), or until the disease started to spread to distant parts of the body (relapsed patients). The Ethics Committee of the HUJ23 and the Research Committee of HTVC approved this study (reference number 24p/2012). We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. According to Spanish law, all patients participating in the study signed a written informed consent.

*Tissue Preparation and Immunohistochemistry*

Two 2-mm diameter cores from $ALNs^{-/+}$ and two from $ALNs^{+/+}$ paraffin-embedded biopsies of the same 72 BC patients were evaluated. For this purpose, pathologists selected two representative areas from the central region of the ALN biopsies using a new haematoxylin-eosin slide. The morphology of the ALN is heterogeneous in human BC; therefore, to extract the tissue cores, the same area was always chosen. The selected area included the lymph node capsule, subcapsular sinus, and cortical, paracortical, and medullar regions. In the case of $ALNs^{+/+}$, the cores were taken from the

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non-metastatic tissue, thereby not including tumour cells. Then, the cores were distributed into the pre-made paraffin recipient block using the Arraymold Kit and were immunohistochemically stained and analyzed for eight immune markers in each patient. In total, 288 cores were studied (72 pairs each of $ALNs^{-/+}$ and $ALNs^{+/+}$ cores) and incorporated into tissue microarrays, as previously described [15–17].

Immune markers were immunohistochemically stained using Autostainer Link 48 (Dako) on each slide with the appropriate primary antibody: T-helper lymphocytes (anti-CD4; clone 4B12; Dako, Santa Clara, CA), cytotoxic T lymphocytes (anti-CD8; clone C8/144B; Dako), natural killer cells (anti-CD57; clone NK1; Zymed, Thermo Fisher Scientific, Waltham, MA), macrophages (anti-CD68; clone KP1; Dako), follicular dendritic cells (DCs) (anti-CD21; clone 1F8; Dako), Langerhans DC (anti-CD1a; clone 010; Dako), interdigitant DCs (anti-S100; polyclonal; Leica Microsystems GmbH, Wetzlar, Germany), and mature DCs (anti-CD83; clone 1H4b; Leica Microsystems GmbH, Wetzlar, Germany). The EnVision FLEX method (Dako), with the chromogen diaminobenzidine as a substrate and haematoxylin counterstaining, was used for the final detection, in accordance with the supplier's instructions and laboratory protocol.

*Slide Digitization and Image Evaluation*

Each stained core slide was transformed into a digital image in Tagged Image File Format (TIFF) at 0.25 mm/pixel resolution and a size of approximately 500 MB, by scanning it at x40 magnification using an Aperio ScanScope XT scanner (Leica Microsystems GmbH, Nussloch, Germany). The 2,304 extracted images (288 cores × 8 immune markers) were stored and classified by the stained immune marker and the corresponding case number.

The digital images were evaluated by automated analysis techniques which had been previously tested for their ability to quantify immunohistochemical markers in cancer [17]. The algorithms were adjusted to suit the specific markers used in the study and were validated by expert pathologists and biologists. These procedures quantify the percentage of positively stained areas of each marker relative to the whole area of the core, with all measurements being expressed in pixels (online suppl. Fig. 2a, b). The final result for each stained marker is the mean percentage of positive pixels of the two cores studied in each patient. In cases where one of the cores was lost due to poor staining, loss of tissue, or poor image quality, we used the information from a single core.

*Statistical Analysis*

Characteristics of the immune populations of the studied cohort were described using the mean and standard deviation for normally distributed data and the median and interquartile range for non-normally distributed data. Differences in concentrations of immune populations were evaluated using unpaired-samples *t* tests and Mann-Whitney U tests (for normally and non-normally distributed data, respectively). Considering the cohort of the 72 patients with nodal metastasis at diagnosis, the immune cell concentrations in different types of ALNs were compared as follows: (1) $ALNs^{+/+}$ from LA vs. TNBC subtypes, (2) $ALN^{+/+}$ vs. $ALN^{-/+}$ in each subtype (LA and TNBC), (3) between $ALNs^{+/+}$ of alive patients or those who died from any cause other than cancer and patients who died specifically from cancer, (4) between $ALNs^{+/+}$ of relapsed and non-relapsed patients. Additionally, the immune populations were compared in the ALNs of patients without nodal metastasis included in the previous studies: (5) $ALNs^{-/-}$ vs. $ALNs^{-/+}$, (6) $ALNs^{-/-}$ vs. $ALNs^{+/+}$. All comparisons are presented graphically in online supplementary Figure 3. Quantitative clinical-pathological variables were evaluated in the same comparisons performed with the immune response. The categorical variables were evaluated using the chi-squared or Fisher's exact test. Kaplan-Meier (KM) curves were calculated from the survival data of the patient cohort, considering all the immune markers. Separate cut-off points using the mean or the median of each immune marker concentration were applied to categorize the patients. Separate KM curves were also derived for LA and TNBC subtypes. Statistical analyses were carried out and KM curves derived using IBM SPSS Statistics (version 21.0; IBM Corp., Armonk, NY, USA).

## Results

*The Immune Profile of $ALNs^{+/+}$ Differs between BC Subtypes*

We first evaluated the differences in the clinical-pathological and immune characteristics of the $ALNs^{+/+}$ between LA and TNBC subtypes (Table 1). Of the demographic and clinical-pathological variables studied, only the histological grade of the tumour, the frequency of relapse and the median TTP turned out to be significantly higher in TNBC than in LA patients. Regarding the IHC analysis, levels of expression of the immune markers CD4, CD68, and CD1a were higher in the $ALNs^{+/+}$ of LA patients, while CD57 was expressed at a significantly lower level in that group than in TNBC patients.

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**Table 1.** Clinical-pathological variables and the percentages of immune populations in the ALNs$^{+/+}$ according to the BC subtype (luminal A or TNBC)

| | Luminal A ($n$ = 46) | TNBC ($n$ = 26) | $p$ value |
|---|---|---|---|
| Age, years | 60.02 (12.85) | 61.00 (13.48) | 0.761[a] |
| Tumour diameter, mm | 22.00 (13.00) | 16.00 (26.75) | 0.248[b] |
| Histological grade, $n$ (%) | | | |
| 1 | 6 (13.0) | 3 (11.5) | **0.001**[c] |
| 2 | 26 (56.5) | 4 (15.4) | |
| 3 | 14 (30.4) | 19 (73.1) | |
| Ki67 degree of expression, $n$ (%) | | | |
| Low | 14 (30.4) | 11 (44.0) | 0.389[c] |
| Medium | 20 (43.5) | 7 (28.0) | |
| High | 12 (26.1) | 7 (28.0) | |
| Node involvement | | | |
| ALNs$^{+}$ | 2.0 (3.0) | 2.5 (4.0) | 0.690[b] |
| Total ALN extracted | 14.0 (9.0) | 16.0 (7.0) | 0.391[b] |
| Menopausal status, $n$ (%) | | | |
| Pre-menopausal | 11 (26.2) | 2 (22.2) | 1.000[c] |
| Post-menopausal | 31 (73.8) | 7 (77.8) | |
| Neoadjuvant treatment, $n$ (%) | | | |
| Yes | 9 (19.6) | 5 (19.2) | 1.000[c] |
| No | 37 (80.4) | 21 (80.8) | |
| Adjuvant treatment, $n$ (%) | | | |
| Yes | 36 (81.8) | 25 (96.2) | 0.139[c] |
| No | 8 (18.2) | 1 (3.8) | |
| Relapse, $n$ (%) | | | |
| Yes | 5 (10.9) | 16 (61.5) | <**0.001**[c] |
| No | 41 (89.1) | 10 (38.5) | |
| Type of relapse, $n$ (%) | | | |
| Distal metastasis | 3 (60.0) | 10 (62.5) | 1.000 |
| Mixed metastasis | 2 (40.0) | 6 (37.5) | |
| Median CSS, months | 112.50 (20.00) | 95.50 (80.00) | 0.240[b] |
| Median TTP, months | 108.50 (24.00) | 43.50 (94.00) | **0.002**[b] |
| ALNs$^{+}$ | | | |
| CD4 | 56.21 (16.75) | 43.69 (18.45) | **0.008**[a] |
| CD8 | 14.63 (5.81) | 12.99 (7.47) | 0.334[a] |
| CD57 | 0.18 (0.28) | 0.40 (0.41) | **0.001**[b] |
| CD21 | 0.90 (2.88) | 0.55 (1.01) | 0.077[b] |
| CD68 | 6.98 (3.37) | 4.71 (4.61) | **0.007**[b] |
| CD1a | 1.81 (2.92) | 0.37 (1.45) | **0.002**[b] |
| S100 | 4.34 (3.77) | 2.49 (3.16) | 0.175[b] |
| CD83 | 0.61 (0.53) | 0.16 (1.11) | 0.062[b] |

Values of $p$ in bold indicate statistically significant differences between the ALNs$^{+/+}$ of the two BC subtypes ($p < 0.05$). Mixed metastasis involves local recurrence and distal metastasis. ALNs$^{+/+}$, metastatic axillary lymph node of metastatic patients; TNBC, triple-negative breast cancer; CSS, cancer-specific survival; TTP, time to progression. [a]Data are presented as the mean (standard deviation) for Student's $t$ test. [b]Data are presented as the median (interquartile range) for the Mann-Whitney U test. [c]Data are presented as the number of patients (percentage) in each category for the chi-squared or Fisher's exact test.

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**Table 2.** Overall percentages of the immune populations in ALNs$^{-}$ from BC patients without nodal metastasis (ALNs$^{-/-}$) and in ALNs$^{-}$ and ALNs$^{+}$ of BC patients with nodal metastasis (ALNs$^{-/+}$ and ALNs$^{+/+}$) divided by BC subtype

| | ALN$^{+/+}$ | ALN$^{-/+}$ | ALN$^{-/-}$ | [a]*p* value | [b]*p* value | [c]*p* value |
|---|---|---|---|---|---|---|
| LA | (*n* = 46) | (*n* = 46) | (*n* = 42) | | | |
| CD4 | 56.21 (16.75) | 55.23 (13.96) | 57.23 (12.73) | 0.909[d] | 0.756[d] | 0.505[d] |
| CD8 | 14.63 (5.81) | 17.23 (8.67) | 12.65 (6.28) | **0.003**[d] | 0.137[d] | **0.008**[d] |
| CD57 | 0.18 (0.28) | 0.16 (0.26) | 0.20 (0.17) | 0.443[e] | 0.522[e] | 0.778[e] |
| CD21 | 0.90 (2.88) | 0.61 (1.36) | 0.76 (1.73) | **0.008**[e] | 0.574[e] | 0.104[e] |
| CD68 | 6.98 (3.37) | 10.30 (5.60) | 7.95 (5.10) | <**0.001**[e] | 0.187[e] | **0.038**[e] |
| CD1a | 1.81 (2.92) | 1.68 (4.38) | 1.60 (2.86) | 0.236[e] | 0.507[e] | 0.273[e] |
| S100 | 4.34 (3.77) | 5.65 (6.37) | 3.04 (3.21) | **0.018**[e] | 0.128[e] | **0.001**[e] |
| CD83 | 0.61 (0.53) | 0.95 (1.18) | 1.13 (1.38) | **0.003**[e] | <**0.001**[e] | 0.660[e] |
| TNBC | (*n* = 26) | (*n* = 26) | (*n* = 30) | | | |
| CD4 | 43.69 (18.45) | 62.89 (14.50) | 63.68 (14.25) | **0.001**[d] | <**0.001**[d] | 0.842[d] |
| CD8 | 12.99 (7.47) | 19.31 (7.07) | 19.75 (6.73) | <**0.001**[d] | <**0.001**[d] | 0.818[d] |
| CD57 | 0.40 (0.41) | 0.73 (0.80) | 0.78 (0.96) | **0.003**[e] | 0.052[e] | 0.626[e] |
| CD21 | 0.55 (1.01) | 1.03 (1.23) | 1.00 (1.19) | 0.167[e] | 0.162[e] | 0.903[e] |
| CD68 | 4.71 (4.61) | 11.17 (5.99) | 9.00 (7.52) | <**0.001**[e] | **0.006**[e] | 0.068[e] |
| CD1a | 0.37 (1.45) | 2.17 (2.49) | 1.44 (4.54) | **0.008**[e] | **0.033**[e] | 0.507[e] |
| S100 | 2.49 (3.16) | 4.44 (4.69) | 3.57 (3.81) | 0.079[e] | 0.529[e] | 0.298[e] |
| CD83 | 0.16 (1.11) | 0.19 (0.57) | 0.25 (0.64) | 0.498[e] | 0.878[e] | 0.748[e] |

Values of *p* in bold indicate statistically significant differences ($p < 0.05$) between the indicated ALNs pairs ([a]*p*, [b]*p*, or [c]*p*). [a]Value from comparison of immune populations between ALN$^{-/+}$ and ALN$^{+/+}$. [b]Value from comparison of immune populations between ALN$^{-/-}$ and ALN$^{+/+}$. [c]Value from comparison of immune populations between ALN$^{-/-}$ and ALN$^{-/+}$. [d]Data are presented as the mean (standard deviation) for Student's *t* test. [e]Data are presented as the median (interquartile range) for the Mann-Whitney U test.

*Matched ALNs from Patients with Axillary Metastasis (ALNs$^{-/+}$ and ALNs$^{+/+}$) Have Different Immune Microenvironment in Both BC Subtypes*

We studied the differences in the levels of the immune markers between ALNs$^{+/+}$ and ALNs$^{-/+}$ from the same patients stratified for the two BC subtypes (Table 2, [a]p). On the one hand, in LA patients, ALNs$^{+/+}$ presented significantly lower levels of CD8, CD68, S100, and CD83 and higher levels of CD21 than ALNs$^{-/+}$. On the other hand, in TNBC patients, ALNs$^{+/+}$ expressed significantly lower levels of CD8, CD68, CD57, CD1a, and CD4 expression than ALNs$^{-/+}$. In both comparisons, the levels of five immune populations differed between ALNs$^{-/+}$ and ALNs$^{+/+}$.

*The Immune Populations in ALNs$^{-}$ from TNBC Patients Differ Much More from ALN$^{+/+}$ than from Luminal A, regardless of Nodal Status*

In order to evaluate the immune profiles in the ALNs further, we compared the immune infiltrate between ALNs$^{-/-}$ (obtained from previous studies mentioned in the Materials and Methods section) and ALNs$^{+/+}$ also stratified by BC subtype (Table 2, [b]p). In LA patients, we found similar concentrations of almost all the immune populations in ALNs$^{-/-}$ and ALNs$^{+/+}$, except for CD83, whose levels were higher in the former ALNs. Furthermore, ALNs$^{+/+}$ from TNBC patients presented lower levels of CD4, CD8, CD68, and CD1a than ALNs$^{-/-}$. Notably, these four immune populations were also statistically significant in the previous comparison (ALNs$^{-/+}$ and ALNs$^{+/+}$). Differences in the levels of CD57 were close to statistical significance. These results suggested that the immune infiltrate may differ more between ALN$^{-/-}$ and ALN$^{+/+}$ in TNBC than in LA patients because we found a higher number of populations to have statistically significant differences between samples in that group. Moreover, unlike in the LA subtype, we found no significant differences between ALNs$^{-/-}$ and ALNs$^{-/+}$ from TNBC patients (Table 2, [c]p).

*Patients Prognoses Prove to Be Related to the Presence of Specific Immune Infiltrates in the ALNs$^{+/+}$ when Considering the Molecular BC Subtype*

We explored the divergence of the clinical-pathological variables and the immune populations in the ALNs$^{+/+}$ of the whole cohort of patients depending on their survival

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**Table 3.** Differences in the clinical-pathological variables and the percentages of the ALNs$^+$ immune populations between patients alive or dead from other causes and patients dead from cancer (CSS) and between non-relapsed and relapsed patients (TTP)

| | Patients alive or dead from other causes ($n$ = 57) | Patients dead from cancer ($n$ = 15) | $p$ value | Non-relapsed patients ($n$ = 51) | Relapsed patients ($n$ = 21) | $p$ value |
|---|---|---|---|---|---|---|
| Age, years | 60.44 (12.57) | 60.13 (14.98) | 0.936[a] | 61.00 (21.00) | 55.00 (32.00) | 0.337[b] |
| Tumour diameter, mm | 20.00 (16.00) | 31.00 (37.00) | 0.145[b] | 21.00 (15.00) | 24.00 (43.00) | 0.392[b] |
| Histological grade, *n* (%) | | | | | | |
| 1 | 8 (14.0) | 1 (6.7) | 0.189[c] | 7 (13.7) | 2 (9.5) | 0.465[c] |
| 2 | 26 (45.6) | 4 (26.7) | | 23 (45.1) | 7 (33.3) | |
| 3 | 23 (40.4) | 10 (66.7) | | 21 (41.2) | 12 (57.1) | |
| Ki67 degree of expression, *n* (%) | | | | | | |
| Low | 20 (35.1) | 5 (35.7) | 0.228[c] | 15 (29.4) | 10 (50.0) | **0.042**[c] |
| Medium | 24 (42.1) | 3 (21.4) | | 24 (47.1) | 3 (15.0) | |
| High | 13 (22.8) | 6 (42.9) | | 12 (23.5) | 7 (35.0) | |
| Menopausal status, *n* (%) | | | | | | |
| Pre-menopausal | 11 (26.8) | 2 (20.0) | 1.000[c] | 9 (22.5) | 4 (36.4) | 0.439[c] |
| Post-menopausal | 30 (73.2) | 8 (80.0) | | 31 (77.5) | 7 (63.6) | |
| Neoadjuvant treatment, *n* (%) | | | | | | |
| Yes | 10 (17.5) | 4 (26.7) | 0.471[c] | 9 (17.6) | 5 (23.8) | 0.532[c] |
| No | 47 (82.5) | 11 (73.3) | | 42 (82.4) | 16 (76.2) | |
| Adjuvant treatment, *n* (%) | | | | | | |
| Yes | 48 (87.3) | 13 (86.7) | 1.000[c] | 42 (85.7) | 19 (90.5) | 0.714[c] |
| No | 7 (12.7) | 2 (13.3) | | 7 (14.3) | 2 (9.5) | |
| Molecular profile, *n* (%) | | | | | | |
| Luminal A | 42 (73.7) | 4 (26.7) | **0.001**[c] | 41 (80.4) | 5 (23.8) | <**0.001**[c] |
| TNBC | 15 (26.3) | 11 (73.3) | | 10 (19.6) | 16 (76.2) | |
| ALN$^+$ | | | | | | |
| CD4 | 51.72 (18.56) | 53.01 (17.36) | 0.821[a] | 52.65 (18.31) | 50.34 (18.33) | 0.644[a] |
| CD8 | 14.09 (6.02) | 14.02 (7.92) | 0.973[a] | 14.56 (6.04) | 12.96 (7.21) | 0.357[a] |
| CD57 | 0.28 (0.32) | 0.27 (0.36) | 0.987[b] | 0.25 (0.30) | 0.29 (0.44) | 0.477[b] |
| CD21 | 0.77 (1.89) | 0.50 (2.61) | 0.483[b] | 1.00 (2.84) | 0.52 (0.88) | 0.111[b] |
| CD68 | 6.48 (3.28) | 5.58 (5.11) | 0.455[b] | 6.81 (3.43) | 5.45 (4.62) | 0.224[b] |
| CD1a | 1.17 (2.96) | 1.41 (2.21) | 0.753[b] | 1.18 (2.96) | 0.93 (2.03) | 0.712[b] |
| S100 | 4.17 (3.00) | 4.68 (3.36) | 0.585[b] | 4.18 (3.01) | 3.66 (3.31) | 0.918[b] |
| CD83 | 0.58 (0.51) | 0.89 (1.22) | 0.187[b] | 0.59 (0.44) | 0.77 (1.14) | 0.612[b] |

Values of $p$ in bold indicate statistically significant differences between the two groups of patients ($p < 0.05$). ALNs$^+$, metastatic axillary lymph nodes; TNBC, triple-negative breast cancer. [a]Data are presented as the mean (standard deviation) for Student's $t$ test. [b]Data are presented as the median (interquartile range) for the Mann-Whitney U test. [c]Data are presented as the number of patients (percentage) in each category for the chi-squared or Fisher's exact test.

(CSS) and relapse (TTP) (Table 3). We observed significant differences in some of the clinical-pathological characteristics, but not in the levels of immune markers. Significantly more TNBC than LA patients died from cancer and/or relapsed. The level of expression of Ki67 in patients who relapsed was significantly greater than in those who did not relapse. The influence of the immune markers on the CSS and TTP of the patients was also evaluated using KM survival curves, overall and for the separate BC subtypes (online suppl. Fig. 4, 5, respectively). Although we did not observe any significant differences when considering all patients together, we found that, separately, TNBC patients with CD4, CD8 or CD83 levels greater than the median in ALNs$^{+/+}$ had worse CSS than patients with lower levels of these markers ($p$ = 0.005, $p$ = 0.028, and $p$ = 0.024, respectively;

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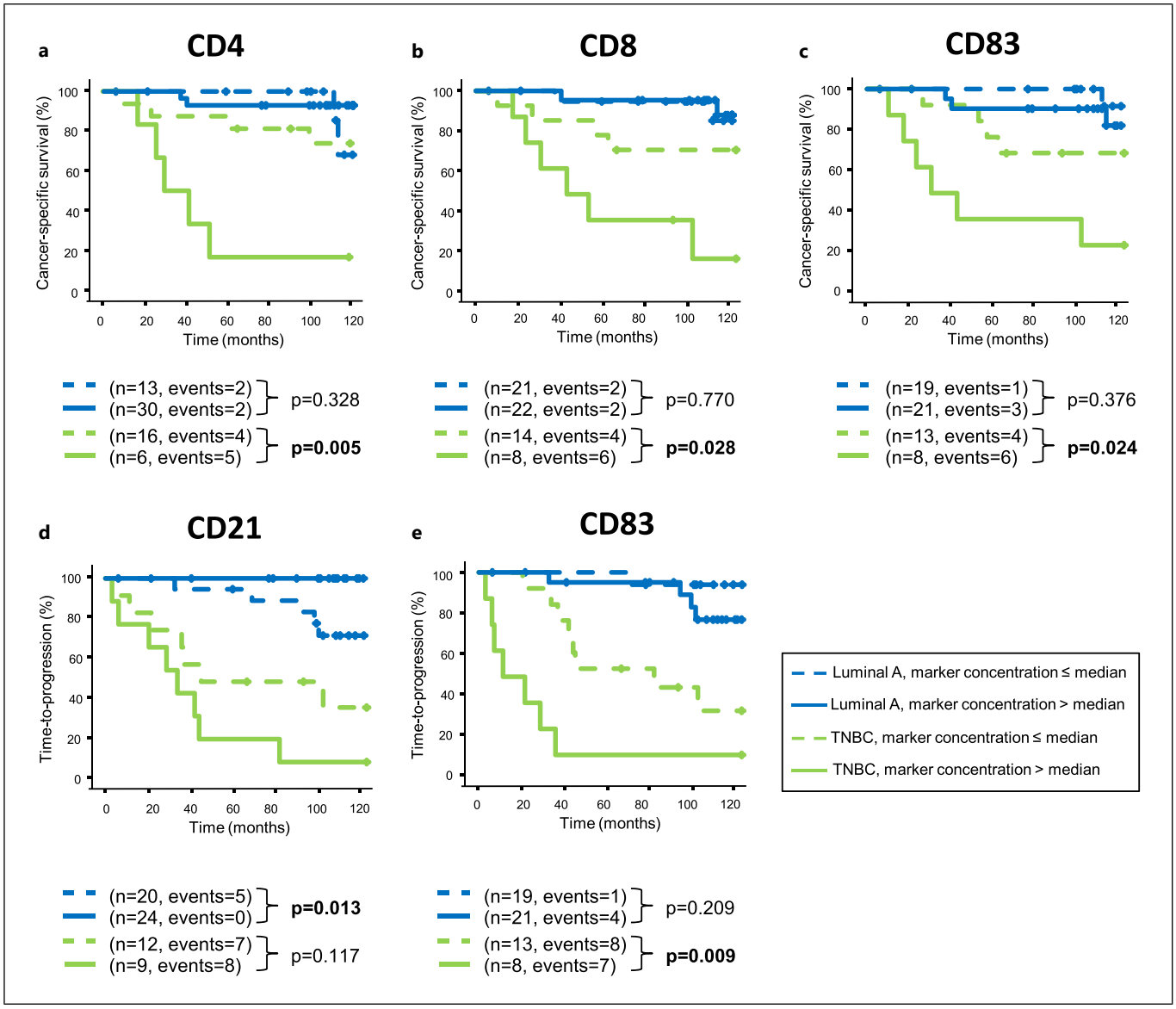



**Fig. 2.** KM curves showing influence of immune marker expression in metastatic axillar lymph nodes ($ALNs^{+}$) on cancer-specific survival (CSS) (**a–c**) and time to progression (TTP) (**d**, **e**) of luminal A vs. triple-negative breast cancer (TNBC) patients. Blue lines indicate patients with luminal A BC; green lines indicate those with TNBC. Dashed and continuous lines indicate patients with marker concentrations less than or equal to, and greater than the median, respectively.

Fig. 2a–c). When studying TTP, as in the case of CSS, no statistically significant differences were observed for the entire cohort. However, we found that LA patients with lower levels of CD21 (less than or equal to the median) in $ALNs^{+/+}$ were more likely to relapse compared to patients with higher levels of this marker ($p$ = 0.013; Fig. 2d). Conversely, TNBC patients with higher levels of CD83 (greater than the median) in $ALNs^{+/+}$ were more likely to relapse compared to patients with lower levels of this marker ($p$ = 0.009; Fig. 2e).

## Discussion

To the best of our knowledge, this is the first study to compare the immune cell components of $ALNs^{+/+}$ in different circumstances: (1) between LA and TNBC subtypes, (2) between matched ALNs for each subtype ($ALNs^{+/+}$ vs. $ALNs^{-/+}$ from the same patient), and (3) between non-matched ALNs for each subtype ($ALNs^{-/-}$ vs. $ALNs^{+/+}$). In addition, this study also evaluates the impact of immune composition in the $ALNs^{+/+}$ on



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patient outcomes, for the two molecular subtypes both combined and separately. Some of the clinical and pathological differences found between LA and TNBC patients and between patients who were alive vs. dead or relapsed vs. non-relapsed were consistent with the results of previous studies [18–22].

Regarding the immune composition in ALNs$^{+/+}$, we found a more immunoactive infiltrate in those from LA patients (a higher proportion of T-helper lymphocytes CD4$^+$, macrophages CD68$^+$, and DC CD1a$^+$ but a lower proportion of CD57$^+$ cells) than in ALNs$^{+/+}$ from TNBC patients. The lower concentrations of immune infiltrates encountered in ALNs$^{+/+}$ from the TNBC subtype might lead to an ineffective anti-tumour response, facilitating cancerous cells entering the blood system and thereby becoming likely to establish distal metastasis. In this context, our group recently reported different concentrations in the immune populations in ALNs$^-$ between patients with and without metastatic ALNs (ALNs$^{-/+}$ vs. ALNs$^{-/-}$). We found that ALNs$^{-/+}$ contain higher concentrations of CD8, CD68, and S100 than ALNs$^{-/-}$ [15]. Taken together, these results reinforce the hypothesis that tumours present in ALNs$^+$ can alter not only their own immune milieu but also that of the unaffected neighbour ALNs by modifying the concentrations of the immune populations. Unexpectedly, when comparing matched ALNs (ALNs$^{-/+}$ vs. ALNs$^{+/+}$ in the same patient) depending on the subtype, we found ALNs$^{-/-}$ presented an immune pattern more similar to that in ALNs$^{+/+}$ rather than to the ALNs$^{-/+}$. In LA patients, the fact that the immunological profile of the ALNs$^{-/+}$ does not resemble that of the ALNs$^{-/-}$ in some immune populations leads us to suppose that the ALN with surrounding metastasis is still able to display immunosurveillance mechanisms. Conversely, once the cancerous cells spread from the tumour and become established in the ALNs, there is a shift from anti-tumour to pro-tumour activity in the ALNs$^{+/+}$, their immune profile being similar to that of an ALN$^{-/-}$. On the other hand, in ALNs$^{-/-}$ of TNBC patients, the proportions of immune infiltrate are almost identical to ALNs$^{-/+}$ but, in both cases, significantly distinct from ALNs$^{+/+}$. Based on these findings and the more immature character of the immune populations found in ALNs$^-$ from TNBC patients, we speculate that these ALNs$^-$ are usually less immunoactive than those of LAs, regardless of their nodal status. This behaviour might explain the aggressiveness of the tumours and the metastatic rate exhibited by the TNBC subtype, which is mainly driven by the immunosuppressive microenvironment present in regional ALNs [23].

Moreover, of all the markers studied, CD8 and CD68 were the only markers with significantly lower expression in ALNs$^{+/+}$ compared with ALNs$^{-/+}$ in LA and TNBC patients. In line with our results, a study evaluating matched ALNs of BC patients reported a significantly lower infiltration density of CD68 in ALNs$^{+/+}$ than in ALNs$^{-/+}$ [24]. Recently, ALNs$^-$ from TNBC patients were also found to contain a higher density of CD1a$^+$ DCs than did ALNs$^+$ [25]. All these findings support previous results indicating a higher immunocompetence of ALNs$^-$ than ALNs$^{+/+}$ and confirm that regional immunosuppressive changes are directly related to nodal metastasis in BC [11, 13].

We subsequently used KM curves to examine whether the levels of immune cells in the ALNs$^{+/+}$ of the whole cohort are related to their survival (CSS) and relapse (TTP) by comparing the median concentrations of immune markers. The overall differences between the median concentrations of the immune cells (Table 3) and the KM curves (online suppl. Fig. 4) were not statistically significant. However, we did find some significant differences upon stratifying the KM curves by BC subtype (online suppl. Fig. 5; Fig. 2), whereby a high level of CD83 expression was a poor prognostic factor for CSS and TTP in the TNBC subpopulation. Conversely, in a study performed in ALNs, Chang et al. found high CD83 levels were correlated with disease-free survival and significantly better survival [26]. The discrepancy between the above findings could be attributed to the distinct BC subtypes of the patients studied: TNBC and LA patients in our cohort and HER2$^+$ and luminal-like subtypes in the report of Chang et al. Furthermore, Iwamoto et al. [27] demonstrated that CD83 was an independent prognostic factor for BC at the intratumor level and reported a significantly better survival rate for patients with a high rather than low frequency of CD83. Accordingly, our previous studies within the same cohort of LA and TNBC patients revealed that higher CD83 DCs levels in ALNs$^-$ and in the primary tumour were protecting factors against relapse and predicting CSS, respectively [16]. All in all, most of these studies suggest that high levels of mature DCs are a good prognostic factor, which directly contradicts the results of the present study. Based on the present and recent findings, we believe the metastatic state of the ALNs might alter the function of mature DCs, whereby the tumour produces an immune-tolerant environment [26, 28, 29]. Moreover, considering the reported link between ALNs$^+$ and immunosuppression responses [11, 12], we hypothesize that mature DCs play an immunosuppressive role in the ALNs$^+$ of TNBC patients. However, further

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characterization of this immune cell subpopulation is necessary if we are to be able to draw definitive conclusions. Our findings are correlative and should not be interpreted as establishing a causal immunosuppressive role of CD83 in ALNs without further functional assays. Given the current ambiguity surrounding the functional role of CD83, future studies incorporating functional assays are required to elucidate its biological significance.

Hence, as previously postulated in the literature, it seems the greater the immune infiltration the better the patient prognosis in either the primary tumour [30] or in ALNs$^{+/+}$ [31], especially in TNBC and HER2$^{+}$ non-luminal BC subtypes. The results we obtained from the ALNs$^{+}$ of TNBC patients display a converse pattern, whereby patients with higher levels of CD4 and CD8 in the ALNs$^{+}$ have worse CSS than those with lower levels. The CD8 marker is expressed in cytotoxic T lymphocytes and is associated with better survival when found in high proportions at the intratumor level in basal-like BC subtypes [32, 33]. Therefore, the roles of CD4 and CD8 remain controversial given the contrasting results of several studies [34–36].

Another significant finding of the present work is the low level of expression of CD21 in ALNs$^{+}$, where it is a factor predictive of relapse in the LA BC subtype. In this context, we previously identified intratumoral CD21 as a risk factor for metastasis in BC independent of subtype [15] and as being significantly more highly expressed in TNBC than in LA [17]. Moreover, in another study, our group found an almost significant association indicating that higher levels of intratumoral CD21 might be a risk factor of relapse ($p = 0.051$) in the same cohort of TNBC and LA BC patients [16]. These findings suggest that CD21 and CD83 behave similarly, although in the opposite direction, since CD21 cells are poor prognostic factors for BC when they occur in the primary tumour, but good prognostic indicators when present in the ALNs$^{+}$. On the one hand, CD83 is a marker of mature DCs but can be expressed in other cells such as activated B and T lymphocytes [37]. On the other hand, CD21 is a marker of follicular DCs but can also be expressed by mature B cells [38]. Therefore, mature DCs and follicular DCs both play major roles regulating the immune responses against BC, especially connecting the innate and the adaptive immune systems [39, 40]. Therefore, to draw a more accurate picture of their involvement in BC progression, further investigation with staining of other functional markers or with functional assays, among other approaches, would be helpful.

One of the limitations of the present study is the fact that we only had information related to ALNs$^{+}$ from a modest-sized cohort of patients with nodal metastasis, which may be a constraint for a powerful statistical analysis. In this regard, a key limitation of this study is the absence of HER2$^{+}$ and Luminal B molecular subtypes. However, including only a few cases of these subtypes may have decreased statistical robustness and reduced statistical power, potentially affecting the reliability and generalizability of the findings. Moreover, although a multivariate analysis adjusting for potential confounders would be of interest, the number of patients in the relevant subgroups is insufficient to support such analysis (patients who died from cancer, $n = 15$; patients who relapsed, $n = 21$). As a general rule, logistic and Cox regression models require at least 10 events per predictive variable to minimize biased estimates [41, 42]. Taking this into account, we could only include one variable in the Cox regression model for survival because we only have 15 patients and two variables in the model for relapsed patients, as there are 21 patients. Despite these limitations, to our knowledge, this is the first study to compare the immune cell components of ALNs$^{+/+}$ under different conditions. Given these preliminary findings, further studies with larger sample sizes are needed to validate the results and allow for adjustment of confounding variables. It is also important to note that our analysis focused exclusively on LA and TNBC subtypes; therefore, the findings may not be directly generalizable to HER2$^{+}$ or Luminal B tumours. Future studies should include these additional subtypes to determine whether the patterns observed here are consistent across the broader spectrum of BC.

Another limitation of the present study is that it should also be noted that the "gold standard" of histopathology is not perfect since not all node material is sampled for histopathology and some micrometastases may not be detected. Although single staining provides limited information and cannot fully characterize the different subtypes or states of activation of certain immune cells, such as natural killer cells or DCs, we noted remarkable differences in several immune markers between groups of patients depending on the BC subtype. Indeed, while almost 90% of the TNBC patients with high levels of CD83 experienced a relapse, none of the LA BC patients with elevated CD21 levels relapsed. These facts reinforce our results and highlight the importance of exploiting the potential of CD83 and CD21 as prognostic immune markers for CSS and TTP, in addition to CD4 and CD8, which also yielded interesting results in larger TNBC patient cohorts.

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## Conclusions

We found differences in the immune concentrations of matched ALNs ($ALNs^-$ vs. $ALNs^+$) from patients with positive nodal status in either LA or TNBC subtypes. In contrast, compared with LA, the levels of immune cells in $ALNs^-$ of the TNBC profile differ much more from $ALNs^+$ than in the LA subtype, regardless of the nodal status (having or not having other $ALNs^+$).

Furthermore, we determined the presence of CD4 and CD8 in $ALNs^{+/+}$ as potential risk factors for CSS and CD83 for both CSS and TTP in TNBC and CD21 as a protective factor for TTP in LA patients. Therefore, we found a converse involvement of $CD4^+$ T-helper lymphocytes, $CD8^+$ cytotoxic T lymphocytes and $CD83^+$ mature DCs vs. $CD21^+$ follicular DCs with BC patient outcome, which at the same time, presented opposite behaviours in $ALNs^{-/+}$ compared with $ALNs^{+/+}$. Hence, in light of our findings, assessing the prognostic value of the immune elements in the $ALNs^{+/+}$ requires us to consider the BC subtypes in the study of $ALNs^{+/+}$ and the nodal status of the nearby ALNs in the immune profiling of $ALNs^{-/+}$. Due to the retrospective design and limited sample size, these findings remain preliminary. Further research in larger, prospective cohorts is needed if we are to better understand the biological rationale behind these findings, which could also be of potential value in anti-tumour therapy based on the immunological pattern and the BC subtype.


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## Statement of Ethics

The Ethics Committee of the HUJ23 and the Research Committee of HTVC approved this study (reference number 24p/2012). We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. According to Spanish law, all patients participating in the study signed a written informed consent.

## Conflict of Interest Statement

The authors declare that they have no conflicts of interest.


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## Author Contributions

Conceptualization: C.L., L.Re., and M.Le.; data curation and writing – review and editing: C.L., L.Re., E.S., and M.Le.; formal analysis: C.L., L.Re., R.B.P., E.S., A.R.-L., and M.Le.; funding acquisition: C.L. and M.Le.; methodology: C.L., L.Re., E.S., N.G.-B., A.F.-C., A.G.N., and M.Le.; resources: R.B.P., M.G.R., G.B., M.Ll., D.M., L.A.-L., S.M.-G., J.F.G.-F., M.A., J.G., and J.A.I.; software: A.K., L.Ro., and J.B.; supervision: M.Le. and C.L.; and writing – original draft: C.L. and L.Re. All authors have read and agreed to the published version of the manuscript.

## Data Availability Statement

The data that support the findings of this study are not publicly available since they contain information that could compromise the privacy of research participants but are available from the corresponding authors (L.Re. and E.S.) upon reasonable request.

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