Breast Cancer

CD68 and CD83 immune populations in non-metastatic axillary lymph nodes are of prognostic value for the survival and relapse of breast cancer patients

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Abstract:	Background: The foremost cause of death of breast cancer (BC) patients is metastasis, and the first site to which BC predominantly metastasizes is the axillary lymph node (ALN). Thus, ALN status is a key prognostic indicator at diagnosis. The immune system has an essential role in cancer progression and dissemination, so its evaluation in ALNs could have significant applications. In the present study we aimed to investigate the association of clinical-pathological and immune variables in the primary tumour and non-metastatic ALNs (ALNs –) of a cohort of luminal A and triple-negative BC (TNBC) patients with cancer-specific survival (CSS) and time to progression (TTP). Methods: We analysed the differences in the variables between patients with different outcomes, created univariate and multivariate Cox regression models, validated them by bootstrapping and multiple imputation of missing data techniques, and used Kaplan–Meier survival curves for a ten-year follow-up. Results: We found some clinical-pathological variables at diagnosis (tumour diameter, TNBC molecular profile and presence of ALN metastasis), and the levels of several immune markers in the two studied sites, to be associated with worse CSS and TTP. Nevertheless, only CD68 and CD83 in ALNs – were confirmed as independent prognostic factors for TTP. Conclusions: The study identified the importance of macrophage and dendritic cell markers as prognostic factors of relapse for BC. We highlight the importance of studying the immune response in ALNs – , which could be relevant to the prediction of BC patients' outcome.
Response to Reviewers:	19th January 2022 Dear Editor-in-Chief Yasuo Miyoshi and Associate Editor Masayuki Nagahashi, We greatly appreciate the thorough job done and the useful feedback given by the editor and reviewers. Please find enclosed our responses to their questions and comments. Following the reviewers advice we have highlighted in red all the substantive changes in the text. We hope the changes fulfil the requirements for the work to be considered for publication in Breast Cancer. As corresponding author, I confirm that all authors have agreed with the submission of the manuscript in its present form. The manuscript is not under consideration elsewhere and I declare that I do not have any business relationships that might lead to a conflict of interest. Thank you in advance for considering our manuscript for publication. Esther Ms. Esther Sauras Colón Pathology Department Molecular Biology and Research Section Hospital de Tortosa Verge de la Cinta C/Esplanetes 14 Tortosa 43500 Spain Phone/Fax: +34 977519104 E-mail: esthersauras.96@gmail.com Reviewer comments: Reviewer 1: Almost all the points raised in the first version of the manuscript have been properly

	addresses by the authors. However, several both minor changes are still required. - Minor comments: 1. About sample size calculation, I agree that we do not need ten or more events per predictor variable as the authors mentioned. I'm not arguing for a small sample size. What needs to be stated is the justification for the time period and sample size of this case collection as a research plan. This is a critical point for a backward-looking study. Our study involved a retrospective cohort of 144 patients diagnosed with invasive breast cancer (88 luminal A and 56 TNBC cases) between 1995 and 2008. The present study is a continuation of other studies previously published by our group and performed in the same cohort. The clinical studies in this cohort started in 2012 and the first publication was the project's protocol in 20141. As the protocol states, the study initially started with a sample size of 100 cases. Most of the samples were from the hospital of Tortosa, which has a small volume of these types of biopsies. So, in order to have at least ten years of follow-up in most of the samples of the cohort, we established this range of time. Moreover, we also needed to increase the sample size to achieve enough samples of the TNBC subtype. We have added an extra explanation of this issue in the methods section in page 6, lines 131-136. 1. López C, Callau C, Bosch R, Korzynska A, Jaén J, García-Rojo M, et al. Development of automated quantification methodologies of immunohistochemical markers to determine patterns of immune response in breast cancer: a retrospective cohort study. BMJ Open 2014;4(8):e005643. 2. About "Luminal B can have some clinical and pathological characteristics that are intermediate between Luminal A and TNBC", we believe that Luminal A, B, TNBC, and HER2 as molecular subtypes have significant biological differences. Especially in lymph node studies such as this one, it is critical to understand why we chose Luminal A and TNBC as selection criteria. If possible, it is recommended to include cases with Luminal B and HER2. We completely agree with the reviewer that studying the immune response in the other breast cancer subtypes would be of great interest. The study of these other subtypes could also provide more information about the differences that might exist in their ALN and about the immune response related to the different subtypes and their direct or indirect implication in the breast cancer patients' outcome. Nevertheless, the present study is the second part of a previous investigation that was focused only on the comparison of Luminal A and TNBC. In this previous study we demonstrated that there is a high number of differences in the immune response in the primary tumour and in the ALN between these two subtypes of BC patients. Therefore, we wanted to go further in the study of these patients and aimed to evaluate which effect the above mentioned differences could have in patients' outcome. Consequently, the current work is focused on these two subtypes. We have added an extra explanation about the selection criteria in the limitations section of the current article in page 16, lines 433-438.
Author Comments:	

19th January 2022

Dear Editor-in-Chief Yasuo Miyoshi and Associate Editor Masayuki Nagahashi,

We greatly appreciate the thorough job done and the useful feedback given by the editor and reviewers. Please find enclosed our responses to their questions and comments. Following the reviewers advice we have highlighted in red all the substantive changes in the text. We hope the changes fulfil the requirements for the work to be considered for publication in *Breast Cancer.*

As corresponding author, I confirm that all authors have agreed with the submission of the manuscript in its present form. The manuscript is not under consideration elsewhere and I declare that I do not have any business relationships that might lead to a conflict of interest.

Thank you in advance for considering our manuscript for publication.

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CD68 and CD83 immune populations in non-metastatic axillary lymph nodes are of prognostic value for the survival and relapse of breast cancer patients

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Keywords: Breast cancer, axillary lymph nodes (ALNs), survival, outcome, immune markers.

List of abbreviations:

ALN, axillary lymph node; *ALN*$^{-}$, non-metastatic axillary lymph node; *ALN*$^{+}$, metastatic axillary lymph node; *AUC*, area under the curve; *BC*, breast cancer; *CI*, confidence interval; *CSS*, cancer-specific survival; *DAB*, diaminobenzidine; *DC*, dendritic cell; *ER*, oestrogen receptor; *HER2*, human epidermal growth factor receptor 2; *HR*, hazard ratio; *HTVC*, Hospital de Tortosa Verge de la Cinta; *Ki67*, proliferation index; *NK*, natural killer; *PR*, progesterone receptor; *ROC*, receiver-operating characteristic; *STROBE*, Strengthening the Reporting of Observational Studies in Epidemiology; *TMA*, tissue microarray; *TNBC*, triple-negative breast cancer; *TTP*, time to progression.

ABSTRACT

Background: The foremost cause of death of breast cancer (BC) patients is metastasis, and the first site to which BC predominantly metastasizes is the axillary lymph node (ALN). Thus, ALN status is a key prognostic indicator at diagnosis. The immune system has an essential role in cancer progression and dissemination, so its evaluation in ALNs could have significant applications. In the present study we aimed to investigate the association of clinical-pathological and immune variables in the primary tumour and non-metastatic ALNs ($ALNs^-$) of a cohort of luminal A and triple-negative BC (TNBC) patients with cancer-specific survival (CSS) and time to progression (TTP).

Methods: We analysed the differences in the variables between patients with different outcomes, created univariate and multivariate Cox regression models, validated them by bootstrapping and multiple imputation of missing data techniques, and used Kaplan–Meier survival curves for a ten-year follow-up.

Results: We found some clinical-pathological variables at diagnosis (tumour diameter, TNBC molecular profile and presence of ALN metastasis), and the levels of several immune markers in the two studied sites, to be associated with worse CSS and TTP. Nevertheless, only CD68 and CD83 in $ALNs^-$ were confirmed as independent prognostic factors for TTP.

Conclusions: The study identified the importance of macrophage and dendritic cell markers as prognostic factors of relapse for BC. We highlight the importance of studying the immune response in $ALNs^-$, which could be relevant to the prediction of BC patients' outcome.

INTRODUCTION

Approximately 2.09 million new breast cancer (BC) cases were diagnosed in 2018, representing 25% of all cancers among females. Furthermore, BC was responsible for more than 620 000 deaths worldwide in 2018, accounting for 15% of all cancer-related deaths among women [1].

Apart from the inherent characteristics of the tumoral cells that determine patients' evolution, the tumour microenvironment has a crucial role in BC progression, metastasis and patient outcome [2, 3]. The immune system, as part of the tumour microenvironment, is of great relevance, and the involvement of the intratumoral immune response in BC patient outcome has been extensively investigated. For instance, tumour-infiltrating lymphocytes are accepted as being strong prognostic factors in human epidermal growth factor receptor 2 (HER2)-positive and triple-negative BC (TNBC) subtypes [4, 5]. The involvement of several types of immune cells of the primary tumour in tumour progression, the clinical response to neoadjuvant chemotherapy, and the relapse and survival of BC patients have also been recognized [6, 7].

Lymph nodes are neuralgic centres of the immune response. Moreover, axillary lymph nodes (ALNs) are the first site where tumoral cells most frequently metastasize [8], and ALN status is a powerful prognostic indicator at diagnosis, signifying lower disease-free and overall survival rates [8-10]. Cancer cells can disseminate to the rest of the body and metastasize to distant sites more easily from ALNs [11]. Such distant metastasis is the leading cause of mortality in BC patients [12].

Regarding the role of the immune response in ALNs of BC patients, few studies have evaluated how the presence of metastasis in ALNs can disrupt their role or composition [13]. Some studies have shown that metastatic ALNs ($ALNs^+$) exhibit an immune tolerance profile [14, 15]. Indeed, it has been suggested that the immune response in ALNs is suppressed even before they metastasize, making them more prone to tumour growth and progression. With respect to the influence of the immune cells of the ALNs on the clinical outcome of patients, almost all studies have focused on evaluating the immune populations of $ALNs^+$. They found that the presence of $PD\text{-}L1^+$ lymphocytes [16], low levels of CD83 dendritic cells (DCs) [14] and low levels of expression of CD4 and CD1a [17] in $ALNs^+$ are associated with poor disease-free survival. It is surprising that, to date, only Khort et al. have found differences in the immune

markers in non-metastatic ALNs (ALNs⁻) with respect to the mean concentrations in patients who relapsed and those who did not [17]. Thus, we are the first to investigate the associations of the immune markers in ALNs⁻ with patient survival and disease progression using Cox regression models and Kaplan–Meier curves.

A recent study by our group demonstrated that the immune populations in ALNs⁻ could have indirect clinical consequences for the outcome of BC patients [18]. Another of our studies, carried out on the same cohort as that featured in the present manuscript, revealed that huge differences exist in the ALNs⁻ between luminal A and TNBC, and that higher levels of immune populations in the ALNs⁻ could be associated with specific BC surrogate subtypes [19]. Luminal A and TNBC could be considered opposite subtypes when considering patient outcome, since they have the best and the worst prognoses, respectively, compared with the other subtypes [20, 21]. The little evidence amassed to date suggests the potential relevance of ALN⁻ immune populations in the progression of BC. Thus, the scope of the present study was to evaluate the immune response of ALNs⁻ in greater detail and to determine whether the immune populations in the ALN⁻ studied in our previous work could be involved in patient outcome 10 years after diagnosis.

MATERIALS AND METHODS

Study design and participants

This study involved a retrospective cohort of 144 patients diagnosed with invasive breast cancer (88 luminal A and 56 TNBC cases) between 1995 and 2008. The criteria used to define Luminal A and TNBC are those included in the current edition of the WHO classification of Breast Cancer when the study was performed [22]. We examined the biopsies from the primary tumours and the ALNs^{-} selected from the archives of the Pathology Department of the Hospital de Tortosa Verge de la Cinta (HTVC) and the Hospital Joan XXIII of Tarragona (Spain). Due to the small volume of these types of biopsies obtained from the HTVC, the main hospital from which most of the samples come, it was necessary to set a broader time period for the sample collection, i.e. between 1995 and 2008. This period allowed obtaining enough samples with representative tissue in the primary tumor and in the ALN, and establishing a ten year follow-up period. All ALN samples were obtained by lymphadenectomy, whereas sentinel lymph node biopsy was an exclusion criterion. Samples identified without enough tissue to obtain a representative sample of the primary tumour or the ALN were discarded. The intensity of the positive internal controls was assessed and those stains that did not reach the expected level of quality were not included in the final statistical analyses. External positive and negative controls were also included in all the immunohistochemical procedures performed. These immunohistochemical quality controls were used because we were aware of the sensitivity of immunohistochemistry to different preanalytical variables and considered it essential to minimize their impact on immunohistochemical measurements. 12.5% of Luminal A and 9.1% of TNBC patients received neoadjuvant therapy, 98.9% of Luminal A and 100% of TNBC patients received adjuvant therapy (hormonal therapy and/or chemotherapy), and 80.7% of Luminal A and 85.5% of TNBC patients received radiotherapy.

The main aim of the present work was to derive Cox regression models that could predict survival or the progression of the disease. With respect to the sample size necessary to obtain a reliable Cox model, Vittinghoff and McCulloch (2007) argued that “problems are fairly frequent with 2–4 events per predictor variable, uncommon with 5–9 events per predictor variable, and still observed with 10–16 events per predictor variable”. Cox models appear to be slightly more

sensitive than logistic regression models. The worst instances of each problem were not severe with 5–9 events per predictor variable and usually comparable to those with 10–16 events per predictor variable [23]. Our final multivariate model for CSS was based on 20 events and three predictor variables (around seven events per predictor variable). The multivariate model for TTP was based on 30 events and five predictor variables (six events per predictor variable). Therefore, based on the Vittinghoff study, the sample sizes for the two models were sufficient for the analysis.

The main focus of this work was to study the association of factors with BC patient outcome, so we evaluated and compared patients' clinical and pathological characteristics, and eleven immune populations from their intratumoral and ALN^- biopsy samples in: (1) patients who were alive or had died from any cause other than cancer *vs.* patients who had died from cancer (cancer-specific survival, CSS); and (2) patients whose disease had begun to spread to other parts of the body *vs.* those whose disease had not spread (time to progression, TTP), over a ten-year follow-up period. Values of the following clinical and pathological variables were collected: age, tumour diameter, histological grade, oestrogen receptor (ER) status, progesterone receptor (PR) status, proliferation index (Ki67), menopausal status, axillary metastasis, administration of adjuvant or neoadjuvant therapy, and molecular profile.

The study was approved by the Ethics Committee of Hospital Joan XXIII de Tarragona (Reference number: 24p/2012) and by the Research Committee of the HTVC. All patients provided their written informed consent to participate in the study and for the use of their biopsy tissues and clinical data, in accordance with Spanish law. We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

Tissue microarray construction and immunohistochemistry

We used paraffin-embedded biopsies to study the eleven types of immune populations in BC primary tumour and $ALNs^-$. Specifically, pathologists selected four representative areas: two from each of two regions (intratumoral and central ALN^-) studied. Afterwards, 2-mm-diameter cylinders were taken from the biopsies. Since the ALNs in BC are heterogeneous, exactly the same region was extracted from all ALNs. The chosen area included the lymph node capsule, subcapsular sinus and cortical, paracortical and medullar regions (Online Resource Fig. S1). B

and T cells areas were represented in the chosen region. The resulting 576 cylinders (144 patients x 2 cylinders x 2 zones) were incorporated into tissue microarrays (TMAs), as described by Callau et al. [24]. Each TMA was sectioned accordingly, yielding eleven slides from each, enabling the study of eleven immune markers. As previously mentioned, TMAs are widely used to study immune responses in breast tumour biopsies [18, 19, 25]. TMA is a high-throughput, cost-effective method that allows samples to be stained under identical conditions. TMAs are considered acceptable for research settings and clinical trials, especially when they involve a large number of samples [26], even though their correspondence with whole-tissue sections is not considered ideal at the diagnostic level.

For the immunohistochemical analysis of each immune marker, the following primary antibodies were used as previously described [18, 19]: CD4 T helper lymphocytes, CD8 cytotoxic T lymphocytes CD57 natural killer (NK) cells, FOXP3 regulatory T cells, CD68 macrophages, CD21 follicular DCs, CD1a Langerhans DCs, CD123 plasmacytoid DCs, S100 interdigitant DCs, CD208 LAMP3 DCs and CD83 mature DCs. Detection was performed with the ENDVISION™ FLEX method (Dako, Santa Clara, CA, USA), following the manufacturer's instructions and using the chromogen diaminobenzidine (DAB), counterstained with haematoxylin, as a substrate.

To acquire digital images of the slides, samples were scanned with an Aperio ScanScope XT scanner at 40X. Images were saved in TIFF format at 0.25-µm/pixel resolution with a mean size of 25 GB per image. Using software developed by members of our group [27, 28], individual cylinders from each slide were automatically separated into single TIFF images to facilitate storage and analysis. Additionally, images were classified according to the stained immune marker and its case number, with a mean size of 500 MB per image. They were evaluated by digital image analysis techniques previously tested for their ability to quantify immunohistochemical markers in cancer [24, 29, 30]. The percentage of positive signal for each immune marker relative to the whole area of the cylinder was used to calculate the amount of a particular immune population. For each patient we calculated the mean of the percentages of the two cylinders for each marker. In the event that one of the cylinders could not be analysed, we used the results of a single cylinder instead of the mean of two.

Statistical analysis

Differences in the immune populations of patients in the intratumoral and the ALN^- regions of the different survival categories (CSS and TTP) were evaluated with Student's t or Mann–Whitney U tests for normally and non-normally distributed data, respectively. Differences in the quantitative clinical-pathological variables (age and tumour diameter) were determined by the same tests. The chi-squared or Fisher's exact test was used to detect differences in the frequencies of categorical clinical-pathological variables (histological grade, ER, PR, Ki67, menopausal status, molecular profile and axillary metastasis) between groups of patients. Values of $p < 0.05$ were considered statistically significant.

To evaluate the association of the variables with CSS and TTP, a univariate Cox regression was fitted for each variable. We then derived a multivariate Cox regression model by backward elimination that estimated hazard ratios (HRs) and 95% confidence interval (CI) for the variables that produced a value of $p < 0.1$ in the univariate analysis. Since the sample size of relapsed and cancer-related death patients was small, we validated the models by two methods: bootstrapping and multiple imputation. The former validation was achieved using the bootstrapping simulation technique available in IBM SPSS Statistics 21.0 (IBM, Armonk, NY, USA), using 10,000 samples and including only those variables that were significant in the multivariate model. The second validation used the multiple imputation of missing data method available in STATA 14.0 (StataCorp LLC, College Station, TX, USA). Ten imputed datasets were used to handle the missing immune marker data, and all the potential predictive variables and outcomes were included in the imputation model. Rubin's rules were used to combine Cox regression model estimates and standard errors.

Furthermore, in order to evaluate the ability of these models to correctly assign patients to the different survival categories (CSS and TTP) we calculated the receiver-operating characteristic (ROC) curves, the area under the curve (AUC), and the sensitivity and specificity for each of the multivariate models and their validations. Statistically significant differences in the AUC between the ROC curves were identified using the test developed by DeLong et al. [31]. Finally, all the immune variables were dichotomized using the mean or median of each immune population, for normally and non-normally distributed data, respectively, as the cut-off. The dichotomization

was performed to check the differences in the distribution of survival times (CSS and TTP) after a ten-year follow-up using the Kaplan–Meier curves and the log-rank test globally, and stratified by surrogate subtypes and ALN metastasis status. All these statistical analyses were performed with IBM SPSS Statistics 21.0.

RESULTS

Clinical, pathological and immune characteristics of patients

Differences in the clinical-pathological characteristics and the mean or median concentration of the immune populations of patients alive/dead from any cause other than cancer (n=124) *vs.* patients dead from cancer (n=20, CSS), and of patients whose disease had progressed (n=30) *vs.* those with disease progression (n=114, TTP) are shown in Table 1.

Immune population concentrations and CSS

We first investigated whether there was an independent association between our variables and CSS by Cox multivariate analysis (Table 2). We found that the tumour diameter, the TNBC molecular profile and the presence of ALN metastasis were directly associated with cancer-related death (Table 2). However, none of the immune markers that appeared to be associated in the univariate models were retained in the final multivariate model (Table 2). The ability of the model to correctly predict patients who were alive or had died from any cause and those who had died from cancer was calculated based on the ROC curve. The AUC of the multivariate model of CSS was quite good 0.80 (95% CI, 0.64-0.91), although its sensitivity was very low (21.4%) and its specificity was 100.0% (Online Resource Fig. S14a). These results show that the model can predict all the patients who will survive or die from any cause other than cancer, but will correctly predict only around one-fifth of patients who will die from cancer. The two validations using the bootstrap and multiple imputation methods confirmed the inclusion of the three variables in the multivariate model (Table 2). The AUC, sensitivity and specificity of the two validations were equal (Online Resource Fig. S14b-c). In both validations, the AUC and the specificity were almost the same as in the original model but the sensitivity continued to be very low.

Apart from the variables associated with the CSS, we also examined whether the concentrations of the different immune populations could affect patient survival. We evaluated the differences in the CSS using the Kaplan–Meier curves between the patients that were greater than compared with less than or equal to the mean or median concentration of each of the immune markers in the primary tumour (Online Resources Fig. S2, Fig. S4 and Fig. S6) and in the ALN^- (Online Resources Fig. S8, Fig. S10 and Fig. S12). Patients were studied globally (Online Resources Fig. S2 and Fig. S8), by surrogate subtype (Online Resources Fig. S4 and Fig. S10) and by nodal status (Online Resources Fig. S6 and Fig. S12). The only statistically significant differences were found in the group of patients with ALN metastasis, whereby patients with a lower intratumoral CD83 concentration had worse survival than those with a concentration greater than the median (Figure 1a). Moreover, when evaluating the overall results, without any stratification, patients expressing higher levels of CD8 and CD57 in $ALNs^-$ also had poorer survival (Figure 2a-b, respectively). The summary of the statistically significant results obtained from the Kaplan–Meier curves are shown in Online Resource Table S1, where there are also the labels for the corresponding figures.

Immune population concentration and TTP

The association of all the variables that were independently associated with the TTP in the multivariate model (tumour diameter, TNBC molecular profile, presence of ALN metastasis, the concentrations of CD68 and CD83 in the ALN^-) were confirmed by the bootstrap technique (Table 3), with the exception of the presence of ALN metastasis, which, in this case, was borderline significant (p=0.057). The validation of the multivariate model by multiple imputation confirmed that the variables associated with disease progression were the tumour diameter, the TNBC molecular profile, the presence of ALN metastasis, and the concentration of CD68 in the ALN^-. However, it also included medium levels of Ki67, and a medium concentration of intratumoral CD68 (Table 3). Nevertheless, CD83 in the ALN^- was dropped from the final model. The AUC and specificity of the original model and of both validations were around 0.9 and 0.85, respectively (Online Resource Fig. S14d-f). As in CSS, results showed a good overall ability to correctly predict patient outcome, and to identify those patients whose disease was not going to progress. The sensitivity ranged from 46% to 61%, and although this is better than for the CSS model, it is not sufficient to be able to reliably predict the patients whose disease will

progress. The low sensitivity in the CSS and TTP models indicates that there is still much to discover in relation to the factors related to patient outcome.

Univariate and multivariate Cox regression models of TTP with only clinical-pathological variables were calculated and validated by bootstrapping (Table 4), and the ROC curve, AUC, sensitivity and specificity were also calculated (Online Resource Fig. S14g-h). In the comparison of our previous bootstrap-validated model of TTP (Online Resource Fig. S14e) with the latter one comprising only the clinical variables (Online Resource Fig. S14h), the DeLong test showed that the AUC of the former model, which included the immune variables, had a statistically significantly better ability to correctly predict outcomes (p=0.007, Online Resource Fig. S14i).

The same set of Kaplan–Meier curves for the TTP of all the immune markers was derived as in the previous section: overall results (Online Resources Fig. S3 and Fig. S9), and results stratified by the two surrogate subtypes studied (Online Resources Fig. S5 and Fig. S11) and by the presence or absence of metastasis in the ALN (Online Resources Fig. S7 and Fig. S13) in the primary tumour (Online Resources Fig. S3, Fig. S5 and Fig. S7) and in the ALN^- (Online Resources Fig. S9, Fig. S11 and Fig. S13). In the primary tumour, patients with higher levels of CD123 and CD208 were found generally to have a shorter TTP in the entire cohort (Figure 1b-c, respectively). Shorter TTP was also observed in luminal A patients with low CD1a levels (Figure 1d), in patients with metastasis with high CD123 or low CD83 levels (Figure 1e and 1g, respectively), and in patients without metastasis with a high level of CD21 (Figure 1f). In the $ALNs^-$, patients with low levels of CD83 (Figure 2d), or with high levels of CD8, CD57 or CD68 (Figure 2c, 2e and 2f, respectively) were found to exhibit shorter TTP in the entire cohort; TNBC patients with high levels of CD68 (Figure 2g), patients with ALN metastasis and low levels of CD83 (Figure 2j), and patients without ALN metastasis and with high levels of CD68, CD8 or CD57 (Figure 2h, 2i and 2k, respectively) were also found to have a shorter TTP. The summary of the statistically significant results obtained from the Kaplan–Meier curves are shown in Online Resource Table S1, where there are also labels for the corresponding figures.

DISCUSSION

Ability of models to predict patient outcome

The clinical and pathological variables (tumour diameter, TNBC molecular profile, axillary metastasis) that prove to be independent risk factors associated with a worse outcome in the present study are widely known to be risk factors for having a shorter CSS or TTP [8, 20]. Apart from these, immune cell markers in the primary tumour are also widely known to be helpful in predicting cancer outcome [7, 32]. Some of the immune variables in our study appeared to be associated with CSS in the univariate analysis, but none of them was independently associated with CSS, either in the primary tumour or in the ALNs$^-$. When evaluating the ability of the model to predict patient outcome, AUC was found to be at least as good as that reported from earlier studies that used several types of variables to predict patient survival [33, 34]. However, these studies did not report values of sensitivity and specificity of the models. Although our CSS model was able to correctly classify the outcome of the patients in 80% of cases, its sensitivity was poor, which means that we are currently unable to correctly predict those patients who will die from the disease. If, in the future, researchers can develop more sensitive models, this will bring us closer to knowing more about the causes associated with patient death. We obtained similar results when we attempted to predict the progression of the illness with the multivariate model of TTP. In this second model, apart from the clinical-pathological factors that were included in the model of CSS, we also found that immune populations of the primary tumour or the ALNs$^-$ were involved. The two most deeply involved populations were those of the CD68 macrophages and the mature CD83 DCs; these are populations whose relation with patient outcome has been observed in other studies [14, 35-37], as discussed in detail below. In this model, the AUC and specificity were better than in the model of TTP that only included clinical variables. It follows from this that models considering the immune response better predict the outcome of BC patients. This demonstrates how the immune populations of the ALNs$^-$ might be involved in the disease progression of BC patients.

Immune populations in the primary tumour and in the ALNs$^-$ related to patient outcome

We evaluated the possible involvement of the immune populations in primary tumour and ALNs$^-$ with a huge number of Kaplan–Meier curves. We have reported only the results indicating

statistically significant differences between the immune populations. However, it is not easy to draw clear conclusions when considering such a large quantity of results as a single group, so we have divided this part of the discussion into two sections. In the first, we will comment on the immune markers that identified statistically significant differences at least three times in different situations. The second section deals with the markers that identified significant differences in only one or two graphical representations. Although we accept that this is an arbitrary distinction, we think that it helps us draw more robust conclusions.

Markers with statistical significance in three or more Kaplan–Meier curves

The first two markers highlighting differences in patient outcome were CD8 T lymphocytes and CD57 NK cells. In both cases, patients with concentrations greater than the median in the $ALNs^{-}$ had worse global CSS and TTP, and in the subgroup of patients stratified as being without metastasis in their ALNs. In our previous work, higher levels of expression of CD8 and CD57 in $ALNs^{-}$ were associated with the presence of the TNBC subtype at diagnosis [19], which is consistent with the TNBC having shorter survival than other BC subtypes. CD57 is a marker present in T and NK cells, so this may be considered a limitation of this single-marker staining. In addition, we could not find any studies of primary tumours or ALNs of BC patients. Hu et al. performed a meta-analysis of 26 published studies of tumour-infiltrating CD57 lymphocytes in solid tumours (non-including BC) and concluded that this immune population can be seen as having a favourable clinical outcome [38]. CD8 is the classic marker for cytotoxic T lymphocytes, which has been widely reported to be an intratumoral prognostic factor associated with better patient survival [39, 40]. However, in the present study we found the opposite outcome when levels of CD57 or CD8 were higher in the $ALNs^{-}$ than reported intratumorally. These differences could be explained by the distinct nature of the microenvironment in the separate compartments.

The third marker to reveal statistically significant differences in four graphs was that of mature CD83 DCs. Differences were identified in the subgroup of patients with ALN^{+} at diagnosis in three of the four graphs. Patients with ALN^{+} and higher concentrations of CD83 in the primary tumour showed better CSS and TPP, but in this subgroup, patients with higher concentrations of CD83 in their $ALNs^{-}$ also had longer TTP. Globally, all patients with higher concentrations of

this marker in their ALNs⁻ also had a longer TPP. In a previous study, our group demonstrated an association between the expression of CD83 in ALNs⁻ with a luminal A BC subtype [19]. This is consistent with our findings, since luminal A patients have better survival than the other BC subtypes. CD83 is a marker of mature DCs commonly found to be associated with a better immune response overall [41], the presence of tumour-free sentinel lymph nodes [42], being downregulated in BC patients relative to healthy subjects [43], and being inversely correlated with the presence of metastasis in the ALN and with the expression of immunosuppressive cytokines in the primary tumour [35]. In addition, in breast [35] and other cancers, such as gastric [44] and gallbladder [45] carcinomas, a lower content of CD83 has been linked to a poorer prognosis or worse survival. In fact, our results are very similar to those of Iwamoto et al. (2003) and Chang et al. (2013), who reported better survival in BC patients with metastasis when there are higher levels of CD83 in the primary tumour [35] and ALNs⁺ [14]; we also found a lower rate of relapse when patients with metastasis had larger amounts of CD83 in the ALNs⁻.

Finally, patients with higher concentrations of CD68 macrophages in their ALNs⁻ showed worse TTP globally, in TNBC and in patients without ALN⁺ at diagnosis. Similar results have been found in studies of CD68 in the intratumoral region. For instance, Ni et al., in a retrospective meta-analysis of non-metastatic BC patients, found that patients with poor relapse-free and overall survival presented higher levels of expression of this macrophage marker [36]. In addition, Yuan et al. also found intratumoral CD68 tumour-associated macrophages to be a significantly unfavourable prognostic factor for TNBC patients [37]. To our knowledge, there have been no studies of the expression of CD68 in ALNs⁻ and its relationship with cancer survival prognosis.

Markers with statistical significance in two or fewer Kaplan–Meier curves

This group comprised a pool of four DC subtypes, most of which were statistically significant in one Kaplan–Meier curve, and all the differences were found exclusively in the immune populations of the primary tumour related to TTP. The various DCs can display pro- and anti-tumour behaviours, all of which are strongly conditioned by the tumour microenvironment [46]. Consistent with our findings, it has been suggested that CD123 stimulates tolerance to tumoral cells, and its presence has been linked to poor prognosis [47, 48]. In the case of CD1a, our

results are in line with the hypothesis of La Rocca et al., who proposed that hormone receptor-positive BCs are likely to have a better prognosis if they have higher levels of CD1a DCs [49]. Furthermore, our previous work revealed CD21 to be associated with ALN metastasis [18]. Reports about CD208 in BC do not concur completely with our results, since Treilleux et al. did not find any significant correlation between CD208 and patient outcome [47], and its presence in sentinel lymph nodes is associated with a lower risk of lymph node metastasis in BC. Nevertheless, CD208 has been linked to poor patient outcome in other cancers [50, 51]. All in all, the results indicate that DCs may have a variety of roles in patient outcome, although more detailed studies are needed to define these more accurately.

LIMITATIONS

We recognize that the single-staining of the immune cells is a limitation of our study. Double- or triple-staining of some immune populations would undoubtedly help in future research to classify exactly the type and phenotype of immune cells, the latter being correlated with patient outcome. The use of other, more advanced techniques, such as spatial transcriptome or mass cytometric imaging, could also help improve the classification of the immune cells. Another limitation of the present study is that it was not possible to evaluate the tumour-infiltrating lymphocytes in accordance with Salgado's criteria [5], since these had not yet been established when the punches of the study were performed, so the selected areas were not suitable for such evaluation. It seems likely that resolving these limitations would boost the predictive capability of the models.

The present article is the continuation of a previous study, where we stated a great number of differences between the ALN^- of luminal A and TNBC subtypes and, consequently, we wanted to further evaluate what kind of impact these immune populations could have in the outcome of BC patients [19]. Nevertheless, further studies are needed to evaluate what kind of implications the immune populations of the ALN^- of luminal B or HER2 subtypes could have in the patients' outcome.

CONCLUSION

This preliminary study has confirmed our previous findings [18, 19], highlighting the possible importance of immune populations of ALN^- to patient outcome, and drawing attention to the need to study their involvement in BC in greater depth. The great impact of the immune response on cancer progression and patient outcome has facilitated the development of new immunotherapeutic strategies, which is certain to lead to major advances. However, preclinical investigations remain crucial if we are to understand the mechanisms underpinning cancer development and patient outcome [52]. Our study highlights two important aspects that need to be addressed in future work: (1) the more specific characterization of the subtypes of immune populations in the ALN^- and in the primary tumour that could have an important role in BC patient outcome; (2) the inclusion of more patients who have died from the disease or whose disease has progressed since their initial diagnosis.

Declarations:

Ethics approval and consent to participate: This study was approved by the Ethics Committee of Hospital Joan XXIII de Tarragona (Reference number: 24p/2012) and by the Research Committee of the HTVC. All patients provided their written informed consent to participate in the study and for the use of their biopsy tissues and clinical data, in accordance with Spanish law. We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

Consent for publication: Yes.

Availability of data and material: Datasets generated and analysed during this study are available from the corresponding authors upon reasonable request.

Competing interests: The authors have no conflicts of interest to declare.

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Author contributions: CL, RB, DM and ML designed the study and selected the sample. CL, JFGF, SMG, JCR, MA, JG, MB, SRV, MLL and ML collected data and reviewed the clinical records. CL, ESC, JCR and ML processed the samples. CL, AK, MGR, GB, LR and JB digitized the samples and developed image analysis procedures. CL, AGN, ESC, AR and ML did the statistical analysis. CL, AGN, ESC and ML designed the figures. All the authors have read and reviewed the article and made significant contributions to the interpretation of the data.

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Figure Legends and Tables

Fig. 1 Kaplan–Meier curves evaluating the influence of the expression in the primary tumour of: (A) CD83 to cancer-specific survival (CSS) of patients by metastasis status; (B) CD123 and (C) CD208 to time to progression (TTP) of all patients; (D) CD1a to TTP of patients by surrogate subtype; (E) CD123, (F) CD21 and (G) CD83 to TTP of patients divided by metastasis status. Blue (concentration less than or equal to the median) and green (concentration greater than the median) lines are based on data from all patients. Black and red lines indicate luminal A and TNBC patients, respectively. Grey and orange lines indicate patients without and with metastasis, respectively. Dashed and continuous lines indicate patients with a marker concentration less than or equal to, or greater than, the median, respectively

Fig. 2 Kaplan–Meier curves evaluating the influence of the expression in the non-metastatic axillary lymph nodes ($ALNs^{-}$) of: (A) CD8, (B) CD57 to cancer-specific survival (CSS) and of (C) CD8, (D) CD83 and (E) CD57 to time to progression (TTP) of all patients; CD68 to TTP of (F) all patients, and patients grouped by (G) surrogate subtype, and (H) metastasis status; and (I) CD8, (J) CD83 and (K) CD57 to TTP of patients grouped by metastasis status. Blue (concentration less than or equal to the median) and green (concentration greater than the median) lines are based on data from all patients. Black and red lines indicate luminal A and TNBC patients, respectively. Grey and orange lines indicate patients without and with metastasis, respectively. Dashed and continuous lines indicate patients with a marker concentration less than or equal to, or greater than, the median, respectively

Table 1 Differences in the clinical-pathological variables and immune markers in the intratumoral and ALN$^-$ regions between patients who died from cancer and patients who were alive, or dead from other causes (CCS); and between relapsed and non-relapsed patients (TTP)

	Patients alive or dead from other causes (n=124)	Patients dead from cancer (n=20)	p	Non-relapsed patients (n=114)	Relapsed patients (n=30)	p
Age (years)	59.9 (11.4)	61.8 (14.5)	0.517†	60.0 (18.0)	57.0 (28.0)	0.555‡
Tumour diameter (mm)	17.0 (13.0)	24.0 (28.0)	**0.025**‡	17.0 (12.3)	22.5 (35.5)	0.392‡
Histological grade						
1	26 (21.0%)	1 (5.0%)	0.069*	25 (21.9%)	2 (6.7%)	0.100*
2	49 (39.5%)	6 (30.0%)		44 (38.6%)	11 (36.7%)	
3	49 (39.5%)	13 (65.0%)		45 (39.5%)	17 (56.7%)	
ER expression						
Positive	76 (61.3%)	6 (30.0%)	**0.017***	75 (65.8%)	7 (23.3%)	**<0.001***
Negative	48 (38.7%)	14 (70.0%)		39 (34.2%)	23 (76.7%)	
PR expression						
Positive	68 (54.8%)	5 (25.0%)	**0.025***	67 (58.8%)	6 (20.0%)	**<0.001***
Negative	56 (45.2%)	15 (75.0%)		47 (41.2%)	24 (80.0%)	
PI (Ki67)						
Low	52 (41.9%)	9 (47.4%)	0.210*	44 (38.6%)	17 (58.6%)	**0.048***
Medium	43 (34.7%)	3 (15.8%)		42 (36.8%)	4 (13.8%)	
High	29 (23.4%)	7 (36.8%)		28 (24.6%)	8 (27.6%)	
Menopausal status						
Pre-menopausal	13 (15.7%)	2 (16.7%)	1.000*	11 (13.4%)	4 (30.8%)	0.211*
Post-menopausal	70 (84.3%)	10 (83.3%)		71 (86.6%)	9 (69.2%)	
Molecular profile						
Luminal A	82 (66.1%)	6 (30.0%)	**0.005***	81 (71.1%)	7 (23.3%)	**<0.001***
TNBC	42 (33.9%)	14 (70.0%)		33 (28.9%)	23 (76.7%)	
Axillary Metastasis						
Positive	57 (46.0%)	15 (75.0%)	**0.030***	51 (44.7%)	21 (70.0%)	**0.024***
Negative	67 (54.0%)	5 (25.0%)		63 (55.3%)	9 (30.0%)	
Neoadjuvant chemotherapy						
Yes	11 (8.9%)	5 (25.0%)	0.050*	10 (8.8%)	6 (20.0%)	0.104*
No	112 (91.1%)	15 (75.0%)		103 (91.2%)	24 (80.0%)	
Adjuvant chemotherapy						
Yes	87 (72.5%)	16 (80.0%)	0.667*	77 (70.0%)	26 (86.7%)	0.109*
No	33 (27.5%)	4 (20.0%)		33 (30.0%)	4 (13.3%)	
Intratumoral						
CD4	1.03 (3.37)	1.32 (2.72)	0.862‡	1.02 (3.26)	1.42 (2.96)	0.621‡
CD8	1.26 (2.47)	1.07 (2.11)	0.704‡	1.26 (2.52)	1.24 (2.04)	0.749‡
CD57	0.12 (0.26)	0.09 (0.31)	0.756‡	0.13 (0.31)	0.07 (0.15)	0.176‡
FOXP3	0.11 (0.20)	0.08 (0.14)	0.451‡	0.11 (0.19)	0.10 (0.16)	0.698‡
CD21	0.003 (0.020)	0.007 (0.028)	0.190‡	0.002 (0.020)	0.011 (0.023)	0.077‡
CD68	2.80 (3.28)	2.94 (3.05)	0.443‡	2.77 (3.27)	3.17 (3.44)	0.233‡
CD1a	0.11 (0.27)	0.09 (0.45)	0.450‡	0.11 (0.28)	0.10 (0.41)	0.564‡
CD123	0.000 (0.053)	0.006 (0.032)	0.669‡	0.000 (0.053)	0.013 (0.039)	0.172‡
S100	0.15 (0.36)	0.20 (0.39)	0.422‡	0.16 (0.36)	0.16 (0.39)	0.796‡
CD208	0.033 (0.101)	0.074 (0.159)	**0.037**‡	0.03 (0.10)	0.07 (0.16)	**0.049**‡
CD83	0.11 (0.22)	0.08 (0.15)	0.078‡	0.11 (0.22)	0.10 (0.14)	0.183‡
ALN$^-$						
CD4	58.82 (13.86)	61.05 (15.60)	0.525†	58.50 (13.68)	61.55 (15.51)	0.309†
CD8	15.18 (9.60)	19.41 (6.95)	**0.030**‡	14.64 (9.20)	19.95 (5.86)	**0.002**‡
CD57	0.25 (0.44)	0.44 (0.95)	0.155‡	0.24 (0.36)	0.62 (0.92)	**0.006**‡
FOXP3	2.08 (1.45)	2.21 (1.99)	0.547‡	2.10 (1.49)	2.08 (1.29)	0.873‡
CD21	0.72 (1.26)	1.18 (1.88)	0.812‡	0.74 (1.29)	1.02 (1.50)	0.936‡
CD68	9.43 (5.58)	11.08 (5.37)	0.099‡	8.92 (5.30)	11.89 (5.11)	**0.002**‡
CD1a	1.68 (3.42)	1.39 (3.00)	0.938‡	1.64 (3.43)	2.11 (3.46)	0.533‡
CD123	1.54 (2.22)	1.90 (2.10)	0.990‡	1.54 (2.18)	1.90 (2.58)	0.425‡
S100	3.93 (4.88)	4.85 (5.94)	0.313‡	4.15 (5.15)	3.95 (5.42)	0.939‡
CD208	0.22 (0.33)	0.28 (0.68)	0.316‡	0.22 (0.33)	0.23 (0.64)	0.618‡
CD83	0.83 (1.25)	0.42 (0.76)	**0.021**‡	0.90 (1.33)	0.30 (0.75)	**<0.001**‡

ALN$^-$=non-metastatic axillary lymph node. CSS=cancer-specific survival. ER=oestrogen receptors. PI=proliferation index. PR=progesterone receptors. TNBC=triple-negative breast cancer. TTP=time to progression.

The data and statistical tests used in the table for the comparisons are the mean (standard deviation) for Student's t test†, the median (interquartile range) for the Mann–Whitney U test‡, and the number of patients (percentage) in each category for the chi-squared or Fisher's exact test*.

Table 2 Univariate and multivariate Cox regression models of CSS, validation of the multivariate model by the bootstrap method, and validation of the univariate and multivariate models by multiple imputation of missing data

	Univariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate with bootstrap HR (95% CI)	p	Univariate with multiple imputation HR (95% CI)	p	Multivariate with multiple imputation HR (95% CI)	p
Age (years)	1.01 (0.98-1.05)	0.476					1.01 (0.98-1.05)	0.491		
Tumour diameter (mm)	1.03 (1.01-1.05)	**0.002**	1.04 (1.01-1.07)	**0.019**	1.02 (1.01-1.04)	**0.003**	1.03 (1.01-1.05)	**0.002**	1.02 (1.01-1.04)	**0.010**
Histological grade										
1	1.0						1.0			
2	3.13 (0.38-25.98)	0.291					3.13 (0.38-25.98)	0.291		
3	6.58 (0.86-50.30)	0.070					6.58 (0.86-50.30)	0.070		
ER expression										
Positive	0.30 (0.12-0.79)	**0.014**					0.30 (0.12-0.79)	**0.014**		
Negative	1.0						1.0			
PR expression										
Positive	0.31 (0.11-0.86)	**0.024**					0.31 (0.11-0.86)	**0.024**		
Negative	1.0						1.0			
PI (Ki67)										
Low	1.0						1.0			
Medium	0.43 (0.12-1.58)	0.201					0.45 (0.12-1.65)	0.226		
High	1.42 (0.53-3.82)	0.486					1.41 (0.52-3.80)	0.495		
Menopausal status										
Pre-menopausal	1.0						1.0			
Post-menopausal	0.86 (0.19-3.93)	0.847					0.78 (0.25-2.39)	0.661		
Molecular profile										
Luminal A	1.0		1.0		1.0		1.0		1.0	
TNBC	3.94 (1.51-10.26)	**0.005**	5.61 (1.77-17.76)	**0.003**	4.51 (1.72-11.83)	**0.002**	3.94 (1.51-10.26)	**0.005**	4.51 (1.72-11.83)	**0.002**
Axillary Metastasis										
Positive	3.46 (1.26-9.55)	**0.016**	5.86 (1.30-26.50)	**0.022**	3.54 (1.26-9.94)	**0.013**	3.46 (1.26-9.55)	**0.016**	3.54 (1.26-9.94)	**0.016**
Negative	1.0		1.0		1.0		1.0		1.0	
Neoadjuvant chemotherapy										
Yes	3.28 (1.19-9.07)	**0.022**					3.28 (1.19-9.07)	**0.022**		
No	1.0						1.0			
Adjuvant chemotherapy										
Yes	1.45 (0.48-4.33)	0.507					1.45 (0.48-4.33)	0.507		
No	1.0						1.0			
Intratumoral										
CD4	0.95 (0.84-1.07)	0.401					0.98 (0.89-1.08)	0.670		
CD8	1.00 (0.88-1.14)	0.996					0.98 (0.85-1.14)	0.836		
CD57	1.03 (0.98-1.09)	0.263					1.03 (0.97-1.08)	0.318		
FOXP3	0.08 (0.00-2.86)	0.166					0.57 (0.07-4.87)	0.606		

CD21c				
> median	1.67 (0.65-4.32)	0.287	1.67 (0.65-4.32)	0.287
≤ median	1.0		1.0	
CD68	1.07 (1.00-1.14)	**0.048**	1.06 (0.99-1.14)	0.078
CD1a	0.92 (0.66-1.27)	0.596	0.82 (0.53-1.27)	0.358
CD123	0.01 (0.00-76.06)	0.304	0.01 (0.00-171.8)	0.315
S100	1.12 (1.01-1.23)	**0.024**	1.06 (0.95-1.19)	0.274
CD208	1.56 (0.82-2.98)	0.176	1.65 (0.90-3.02)	0.103
CD83	0.02 (0.00-1.78)	0.087	0.04 (0.00-3.93)	0.156
ALN$^-$				
CD4	1.01 (0.98-1.04)	0.535	1.01 (0.98-1.04)	0.489
CD8	1.04 (0.99-1.09)	0.126	1.04 (0.99-1.09)	0.156
CD57	1.30 (0.67-2.55)	0.436	1.35 (0.70-2.62)	0.374
FOXP3	1.18 (0.83-1.68)	0.345	1.14 (0.80-1.64)	0.463
CD21	1.01 (0.81-1.27)	0.916	1.02 (0.83-1.26)	0.843
CD68	1.10 (1.01-1.20)	**0.032**	1.10 (1.01-1.20)	**0.034**
CD1a	1.00 (0.88-1.14)	0.997	0.99 (0.86-1.14)	0.893
CD123	1.12 (0.93-1.34)	0.228	1.13 (0.95-1.34)	0.165
S100	1.07 (0.96-1.20)	0.229	1.05 (0.93-1.18)	0.444
CD208	1.77 (0.66-4.72)	0.253	1.59 (0.58-4.38)	0.370
CD83	0.46 (0.21-0.98)	**0.045**	0.48 (0.22-1.03)	0.058

ALN$^-$=non-metastatic axillary lymph node. c=variables categorized using the median as cut-off. CI=confidence interval. CSS=cancer-specific survival. ER=oestrogen receptor. HR=hazard ratio. PI=proliferation index. PR=progesterone receptor. TNBC=triple-negative breast cancer.

Table 3 Univariate and multivariate Cox regression models of TTP, validation of the multivariate model by the bootstrap method, and validation of the univariate and multivariate models by multiple imputation of missing data

	Univariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate with bootstrap HR (95% CI)	p	Univariate with multiple imputation HR (95% CI)	p	Multivariate with multiple imputation HR (95% CI)	p
Age (years)	1.00 (0.96-1.03)	0.779					0.99 (0.96-1.03)	0.754		
Tumour diameter (mm)	1.02 (1.00-1.04)	**0.021**	1.04 (1.01-1.07)	**0.018**	1.02 (1.00-1.05)	**0.036**	1.02 (1.00-1.04)	**0.021**	1.03 (1.00-1.05)	**0.016**
Histological grade										
1	1.0						1.0			
2	2.97 (0.66-13.42)	0.156					2.97 (0.66-13.42)	0.156		
3	4.68 (1.08-20.26)	**0.039**					4.68 (1.08-20.26)	**0.039**		
ER expression										
Positive	0.19 (0.08-0.44)	**<0.001**					0.19 (0.08-0.44)	**<0.001**		
Negative	1.0						1.0			
PR expression										
Positive	0.21 (0.09-0.51)	**0.001**					0.21 (0.09-0.51)	**0.001**		
Negative	1.0						1.0			
PI (Ki67)										
Low	1.0						1.0		1.0	
Medium	0.28 (0.10-0.84)	**0.023**					0.30 (0.10-0.90)	**0.032**	0.25 (0.08-0.75)	**0.014**
High	0.79 (0.34-1.83)	0.584					0.80 (0.35-1.86)	0.612	0.64 (0.27-1.52)	0.309
Menopausal status										
Pre-menopausal	1.0						1.0			
Post-menopausal	0.39 (0.12-1.26)	0.115					0.50 (0.20-1.28)	0.147		
Molecular profile										
Luminal A	1.0		1.0		1.0		1.0		1.0	
TNBC	6.42 (2.75-14.99)	**<0.001**	4.30 (1.36-13.61)	**0.013**	3.67 (1.39-9.72)	**0.003**	6.42 (2.75-14.99)	**<0.001**	5.51 (2.28-13.29)	**<0.001**
Axillar Metastasis										
Positive	2.66 (1.22-5.81)	**0.014**	7.45 (1.59-34.82)	**0.011**	2.53 (1.09-5.90)	**0.057**	2.66 (1.22-5.81)	**0.014**	3.49 (1.43-8.51)	**0.006**
Negative	1.0		1.0		1.0		1.0		1.0	
Neoadjuvant chemotherapy										
Yes	2.37 (0.97-5.81)	0.059					2.37 (0.97-5.81)	0.059		
No	1.0						1.0			
Adjuvant chemotherapy										
Yes	2.51 (0.88-7.19)	0.087					2.51 (0.88-7.19)	0.087		
No	1.0						1.0			
Intratumoral										
CD4	0.97 (0.90-1.05)	0.504					0.99 (0.93-1.06)	0.856		
CD8	0.98 (0.86-1.11)	0.699					0.99 (0.88-1.12)	0.928		
CD57	0.63 (0.26-1.55)	0.314					0.89 (0.69-1.15)	0.350		
FOXP3	0.51 (0.09-2.89)	0.445					0.98 (0.25-3.80)	0.973		

CD21c										
> median	2.22 (1.00-4.94)	0.051					2.22 (1.00-4.94)	0.051		
≤ median	1.0						1.0			
CD68	1.07 (1.01-1.13)	**0.025**					1.07 (1.02-1.13)	**0.011**	1.10 (1.02-1.18)	**0.012**
CD1a	0.91 (0.68-1.21)	0.499					0.89 (0.66-1.20)	0.435		
CD123	0.18 (0.00-24.93)	0.494					0.13 (0.00-24.63)	0.438		
S100	1.12 (1.01-1.24)	**0.035**					1.06 (0.94-1.20)	0.321		
CD208	1.32 (0.72-2.43)	0.366					1.45 (0.84-2.51)	0.179		
CD83	0.47 (0.08-2.73)	0.401					0.45 (0.08-2.55)	0.362		
ALN$^-$										
CD4	1.01 (0.99-1.04)	0.283					1.01 (0.99-1.04)	0.285		
CD8	1.05 (1.01-1.09)	**0.010**					1.05 (1.01-1.09)	**0.017**		
CD57	1.79 (1.13-2.84)	**0.014**					1.81 (1.15-2.87)	**0.011**		
FOXP3	1.04 (0.76-1.42)	0.829					1.01 (0.74-1.39)	0.947		
CD21	1.01 (0.84-1.21)	0.945					1.01 (0.85-1.21)	0.875		
CD68	1.13 (1.06-1.21)	**<0.001**	1.12 (1.02-1.23)	**0.017**	1.11 (1.03-1.19)	**0.005**	1.13 (1.05-1.21)	**0.001**	1.08 (1.00-1.16)	**0.045**
CD1a	1.03 (0.93-1.14)	0.568					1.02 (0.92-1.13)	0.704		
CD123	1.13 (0.98-1.30)	0.100					1.14 (0.99-1.30)	0.065		
S100	1.02 (0.91-1.13)	0.774					0.99 (0.89-1.11)	0.921		
CD208	1.47 (0.64-3.38)	0.365					1.35 (0.57-3.18)	0.494		
CD83	0.33 (0.16-0.68)	**0.003**	0.33 (0.13-0.84)	**0.020**	0.49 (0.24-0.99)	**0.015**	0.36 (0.18-0.73)	**0.005**		

ALN$^-$=non-metastatic axillary lymph node. c=variables categorized using the median as cut-off. CI=confidence interval. ER=oestrogen receptor. HR=hazard ratio. PI=proliferation index. PR=progesterone receptor. TNBC=triple-negative breast cancer. TTP=time to progression.

Table 4 Univariate and multivariate Cox regression models of TTP using only clinical-pathological variables and validation of the multivariate model by the bootstrap method

	Univariate HR (95% CI)	p	Multivariate HR (95% CI)	p	Multivariate with bootstrap HR (95% CI)	p
Age (years)	1.00 (0.96-1.03)	0.779				
Tumour diameter (mm)	1.02 (1.00-1.04)	**0.021**				
Histological grade						
1	1.0					
2	2.97 (0.66-13.42)	0.156				
3	4.68 (1.08-20.26)	**0.039**				
ER expression						
Positive	0.19 (0.08-0.44)	**<0.001**				
Negative	1.0					
PR expression						
Positive	0.21 (0.09-0.51)	**0.001**				
Negative	1.0					
PI (Ki67)						
Low	1.0					
Medium	0.28 (0.10-0.84)	**0.023**				
High	0.79 (0.34-1.83)	0.584				
Menopausal status						
Pre-menopausal	1.0					
Post-menopausal	0.39 (0.12-1.26)	0.115				
Molecular profile						
Luminal A	1.0		1.0		1.0	
TNBC	6.42 (2.75-14.99)	**<0.001**	7.30 (3.09-17.24)	**<0.001**	7.56 (3.22-17.76)	**<0.001**
Axillar Metastasis						
Positive	2.66 (1.22-5.81)	**0.014**	3.30 (1.49-7.31)	**0.003**	3.43 (1.56-7.54)	**0.001**
Negative	1.0		1.0		1.0	
Neoadjuvant chemotherapy						
Yes	2.37 (0.97-5.81)	0.059				
No	1.0					
Adjuvant chemotherapy						
Yes	2.51 (0.88-7.19)	0.087				
No	1.0					

ALN^{-}= non-metastatic axillary lymph node. CI=confidence interval. ER=oestrogen receptor. HR=hazard ratio. PI=proliferation index. PR=progesterone receptor. TNBC=triple-negative breast cancer. TTP=time to progression.

Figure 1

Primary tumour

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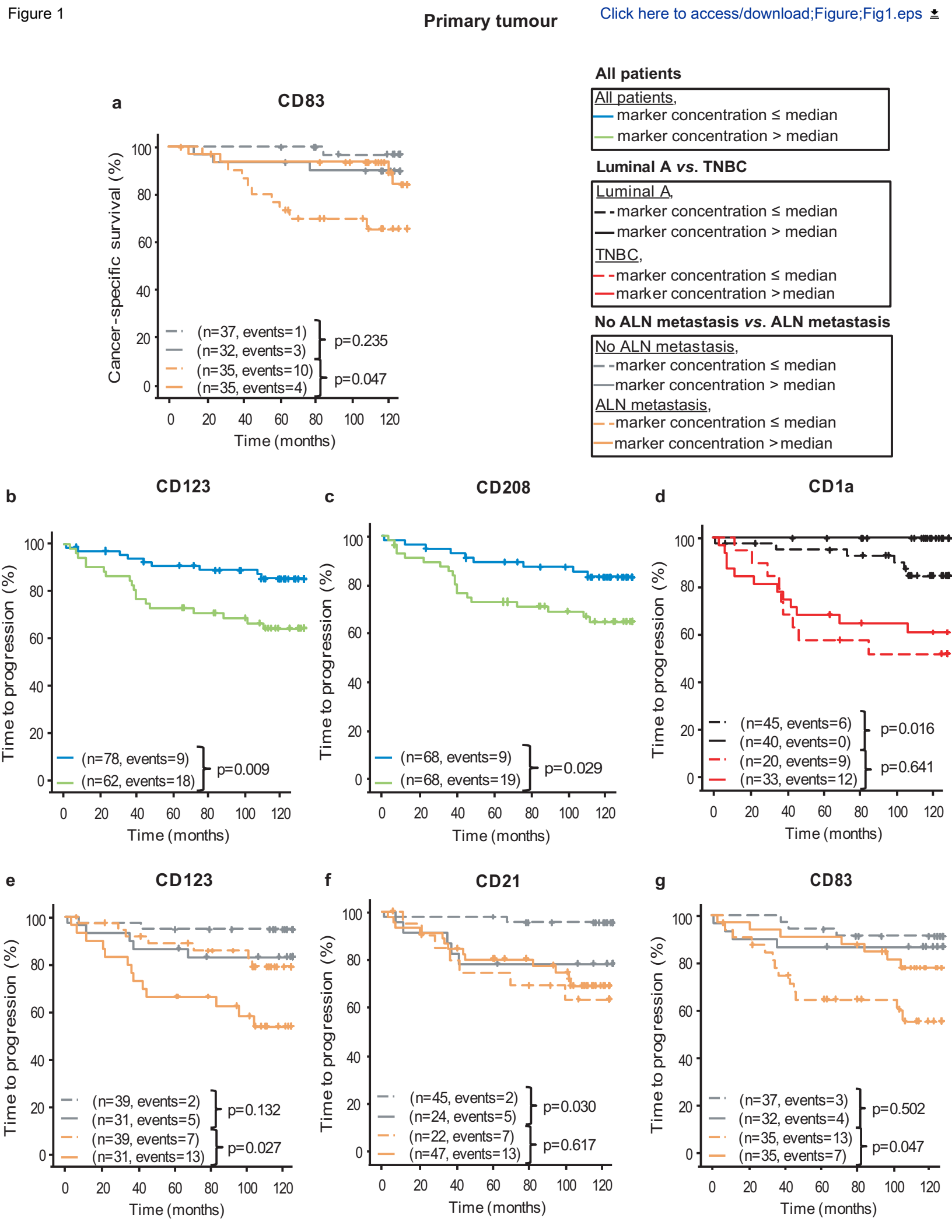
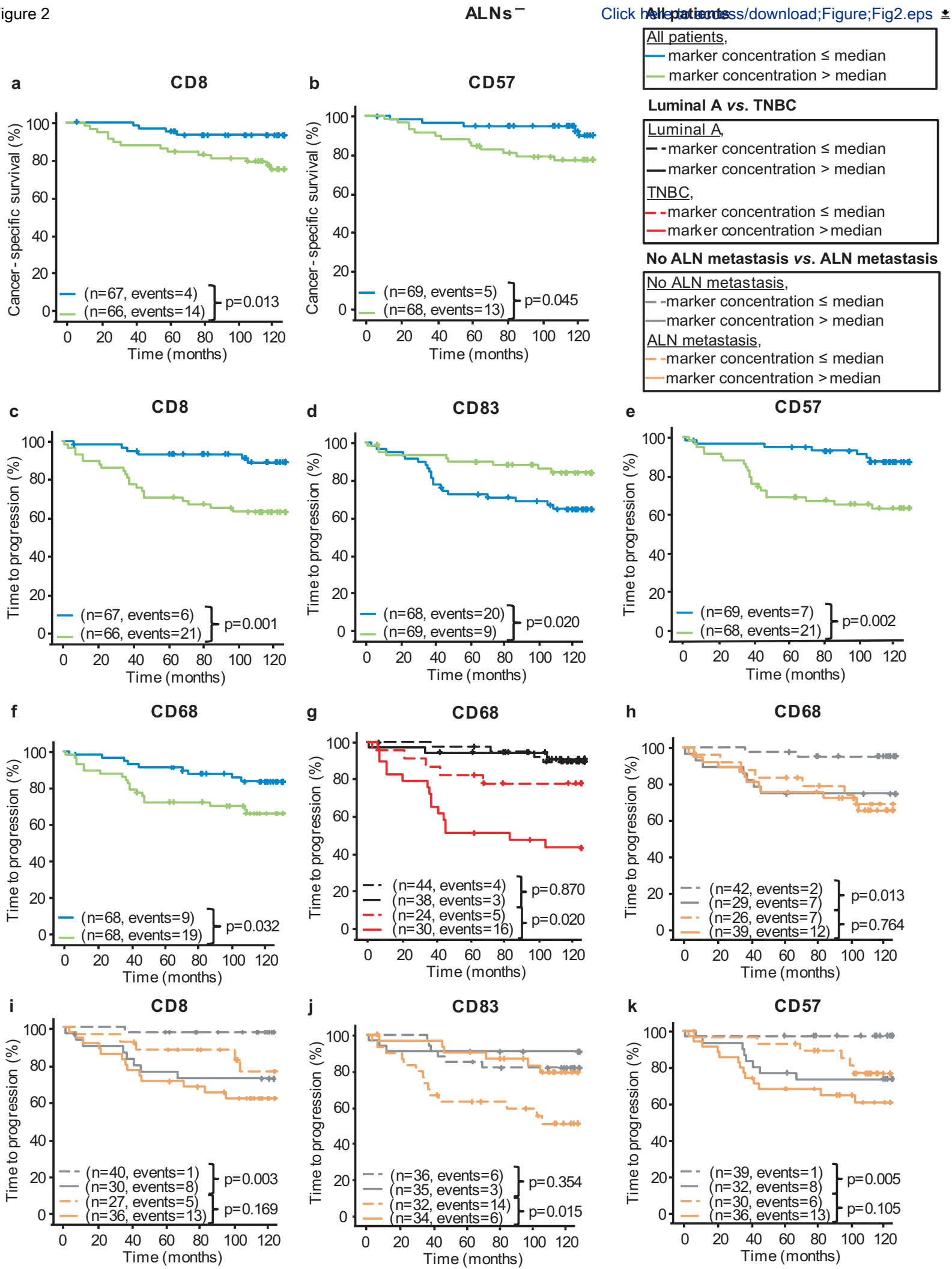


Figure 2



30th July 2021

Dear Editor-in-Chief Yasuo Miyoshi,

We are submitting our article, *CD68 and CD83 immune populations in non-metastatic axillary lymph nodes are of prognostic value for the survival and relapse of breast cancer patients* by López et al., for consideration for publication in the *Breast Cancer*, since we hope that our work will encourage researchers to study the breast cancer immune response from a different point of view.

The submitted article could be considered as a continuation of our previous work reported in our recent papers (Lopez C et al. The immune response in non-metastatic axillary lymph nodes is associated with the presence of axillary metastasis and breast cancer patient outcome. Am J Pathol 2020; and Lopez C et al. Differences in the immune response of the nonmetastatic axillary lymph nodes between triple-negative and luminal A breast cancer surrogate subtypes. Am J Pathol 2021), in which we studied the immune response factors in primary tumours and non-metastatic axillary lymph nodes ($ALNs^-$) associated with breast cancer (BC). These papers present indirect evidence of how the immune response of the $ALNs^-$ could affect patients' outcome. The present submission goes further by showing how specific immune populations could be directly involved in BC patients' outcome.

BC is the most frequent cancer in women, and a leading cause of cancer deaths. Four main BC surrogate subtypes with distinct morphological features and clinical behaviors have been described. In addition, determining the status of the axillary lymph nodes (ALNs) has been one of the greatest advances in breast cancer prognosis and management. ALNs are one of the first regions where BC metastasis establishes, and their infiltration by tumoral cells is a bad prognostic factor whose importance varies between BC subtypes.

The immune response in BC, as part of its tumour microenvironment, has been demonstrated to be involved in BC patients' outcome. The ALNs are particularly important in antitumoral immunity, and suppression of the immune response of the ALNs is provoked by tumoral invasion, nevertheless, little research has been carried out into the immune response in the ALNs, and even less into $ALNs^-$. Given that BC subtypes greatly differ in their prognosis, that ALNs status, metastatic spread and the immune response are decisive in BC, and the immune response is involved in tumor progression even in the $ALNs^-$, we decided to investigate the association of clinical-pathological and immune variables both in the primary tumour and $ALNs^-$ of a cohort of luminal A and TNBC patients with cancer-specific survival (CSS) and time-to-progression (TTP). This is the first study, to our knowledge, investigating the associations of the immune markers in $ALNs^-$ with patients' survival or relapse using Cox regression models and Kaplan-Meier curves.

Our study remarks the importance of macrophages and dendritic cells (DCs) markers as prognostic factors for BC. In particular, CD68 macrophages and CD83 DCs in $ALNs^-$ were found to be independent prognostic factors for TTP. Our results highlight the need of studying the immune response in $ALNs^-$, which could be of relevancy for predicting BC patients' outcome.

As corresponding author, I confirm that all my co-authors have agreed with the submission of the manuscript in its present form. Our manuscript comprises original unpublished work and is not under consideration for publication elsewhere. I declare that none of the authors has any business or personal relationship that might represent a conflict of interest.

Thank you in advance for considering our manuscript.

Sincerely,

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