We have investigated the distribution of chemokine receptor 4 (CXCR4) and CD8-positive, tumour-infiltrating T lymphocytes (CD8+ TILs) in breast cancer subtypes and explored the relationship between them and the well-established conventional prognostic markers, including axillary lymph node involvement. A total of 250 breast cancer patients were included in the study. The patients were separated into luminal A+B, HER2 enriched/overexpressed (HER2+), and triple-negative, on the basis of their staining characteristics, via conventional staining methods. Immunohistochemical (IHC) staining for CXCR4 and CD8+ TILs were performed on the archival tissues from each patient. With increasing intensity of CXCR4 staining, there was a higher incidence of lymph node metastasis (p < 0.01). Similarly, there was a positive correlation between the primary tumour size, HER2+ subtype, lymphovascular invasion, and axillary lymph node involvement. Dense lymphocytic infiltration was observed in HER2+ and triple-negative patients. No correlation between CD8+ TILs in all sites and breast cancer subtypes was discovered. A reverse correlation was discovered with CD8+ TILs stained only intratumorally and CXCR4 expression.

In conclusion, lymph node involvement correlates with higher CXCR4 expression in all breast cancer subtypes. Conversely, no such correlation is found with CD8+ TILs.

Key words: breast cancer, breast cancer subtype, CXCR4, tumour-infiltrating lymphocytes.
implications of CD8+ TILs in breast cancer. In some cases, the intense presentation of CD8+ TILs in the tumour microenvironment translated to complete pathological response and better outcome after neoadjuvant chemotherapy [3, 4, 5]. Furthermore, in some clinical trials, the presence of PDL1 receptor overexpression led to better response with immune checkpoint inhibitors. These findings raise expectations in future trials with immune-mediated treatments [6].

Chemokines have also played a major role in the homing of cancer cells and enhancing their metastatic potential. In this regard, chemokine receptor 4 (CXCR4) plays a major role in breast cancer. No CXCR4 expression is discovered in normal mammary cells; whereas, intense expression of these receptors has been reported in breast cancer cells, correlating with their ability to metastasise to both regional and distant sites [7, 8, 9].

Encouraged by these observations, we decided to investigate the relationship between CXCR4 and CD8+ TILs with respect to regional lymph node involvement in each breast cancer subtype. Additionally, we compared them with each other as well as other known prognostic markers.

Material and methods

Study patients

The study included 250 consecutive patients who underwent either mastectomy or breast-conserving surgery with axillary nodal evaluation either by sentinel lymph node biopsy alone or sentinel lymph node biopsy followed by axillary nodal dissection, between January 2011 and June 2015.

The original pathology reports of all the patients included: patient age, histologic type, primary tumour size, histologic grade in accordance with modified Bloom-Richardson criteria, status of lymphovascular invasion, and status and extent of axillary nodal involvement. The reports also included ER, PR, HER2 expression, and Ki-67 staining properties, of the malignant cells were taken into consideration and were graded as follows: 0 for none, 1 for 1 to 25% cells, 2 for 26% to 75% cells, and 3 for > 75% cells staining positively (Fig. 1). Whereas, for the evaluation of tumour infiltrating lymphocytes, positive nuclear staining for CD8 was assessed in three locations; specifically, within the concentrated tumour cell nests (intratumoral), the adjacent stroma (CD8-positive cells within one tumour cell diameter of the tumour), and the distant stroma (CD8-positive cells more than one tumour cell diameter away; Fig. 2) [1, 11]. At each location, the most prominent staining spot was taken into consideration for assessment.

For CXCR4, cytoplasmic and cell membrane staining properties of the malignant cells were taken into consideration and were graded as follows: 0 for none, 1 for IHC or had HER2/neu gene amplification by FISH. HER2 was considered positive when the IHC staining score was 0 or 1+, and positive when 3+ FISH analysis was performed only for 2+ score to confirm gene amplification.

Pathology assessment

Paraffin-embedded blocks from each patient were processed accordingly and stained with hematoxylin and eosin (HE) for histological evaluation. All of the IHC stainings was performed with a Dako Autostainer 48 Link (Dako, Denmark) for ER (Dako, ready to use), PR (Dako, ready to use), HER2/neu (Dako, dilution 1/400), and Ki-67 (Dako, ready to use) as part of the routine evaluation. Two separate tissue blocks were removed from archival tissues belonging to each corresponding patient, to be analysed for CXCR4 and CD8 expression characteristics. Two three-micron-thick slices were made from each block for IHC staining of CXCR4 (Spring Bioscience) at 1 : 50 dilution and CD8 (Novocasta, Wetzlar, Germany) at 1 : 20 dilution, using monoclonal mouse antibodies via an automatic immunohistochemical staining device (Dako Autostainer 48 Link (Dako, Denmark)). Tonsilar tissue staining for CD8, and intratumoural endothelial cells for CXCR4, were used as positive controls. For both CXCR4 and CD8, the evaluation of the respective staining properties was done by two pathologists using a light microscope, independently, and without any knowledge of the clinical characteristics of the patients. A third pathologist intervened when a disagreement occurred between the two preceding pathologists. The final decision was based on two out of three pathologists agreeing with the assessment.

Statistical analysis

Data obtained from this study was entered to the Statistical Package for Social Sciences (SPSS) data analysis
Results

This study included the analysis of archival tissues from 250 patients. The median patient age was 56 years (range 30-99). Tumour size and the status of regional lymph node metastasis were defined in accordance with TNM staging criteria established by the American Joint Committee on Cancer (AJCC). The median tumour size was 2.5 cm (range 0.6-12). Tumour size In 106 patients was < 2 cm; in 133 patients 2-5 cm, and in 11 patients > 5 cm.

Out of 250 patients, 188 patients (75.2%) were luminal A + B, 44 patients (17.6%) were HER2 positive, and 18 patients (7.2%) were triple negative. Furthermore, when patients were analysed with respect to metastasis to axillary lymph nodes, 108 patients (43.2%) had no metastasis, and 142 patients (56.8%) demonstrated metastasis. In 77 patients (30.8%) the number of metastatic lymph nodes was...
one to three, and in 65 patients (26%) more than three lymph nodes showed metastatic involvement.

When the relationship between CXCR4 and axillary lymph node metastasis was analysed, a statistically significant correlation was discovered between higher CXCR4 expression and axillary lymph node involvement. With increasing CXCR4 staining intensity, axillary lymph node involvement became more prevalent (p < 0.01; Table I). Conversely, in 60 patients who did not have axillary lymph node involvement, the CXCR4 staining was negative. Nevertheless, there was no statistically meaningful relationship between CXCR4 expression and breast cancer subtypes.

We observed dense lymphocytic infiltration in HER2+ and triple negative patients with HE staining; however, there was no correlation between CD8+ TILs, albeit in different locations (intratumoural, adjacent stroma, and distant stroma) and breast cancer subtypes, axillary lymph node metastasis, lymphovascular invasion, and ER and PR expression. Notwithstanding, there was an increasing correlation between the tumour size and CD8+ TILs infiltration at intratumoural and adjacent stromal sites. In tumours with high Ki-67 proliferation index, more intense infiltration of CD8+ TILs was discovered intratumoural, adjacent stroma, and distant stromal sites (p < 0.05). A reverse correlation was discovered between decreasing CXCR4 staining and CD8+ TILs, only at the intratumoural site (r: −0.136).

In 120 patients (48%) lymphovascular invasion was observed, whereas in 130 (52%) patients no lymphovascular invasion was discovered. There was a statistically significant correlation between lymphovascular invasion and axillary lymph node involvement. Among 130 patients who did not show lymphovascular invasion, 92 (70.8%) had no axillary lymph node involvement. Conversely, in 120 patients who showed lymphovascular invasion, 104 (86.6%) demonstrated axillary lymph node involvement in one or more lymph nodes (p < 0.01).

<table>
<thead>
<tr>
<th>CXCR4 expression</th>
<th>Count</th>
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</thead>
<tbody>
<tr>
<td>&gt; 75%</td>
<td>17</td>
<td>85.0%</td>
</tr>
<tr>
<td>26-75%</td>
<td>18</td>
<td>78.3%</td>
</tr>
<tr>
<td>1-25%</td>
<td>49</td>
<td>55.1%</td>
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<tr>
<td>Negative</td>
<td>58</td>
<td>49.2%</td>
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<tr>
<td>Total</td>
<td>142</td>
<td>56.8%</td>
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</table>

<table>
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<tr>
<th>Tumour type</th>
<th>HER 2+</th>
<th>Luminal B</th>
<th>Luminal A</th>
<th>Triple Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
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<td>43</td>
<td>7</td>
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<td>% within tumour type</td>
<td>70.5%</td>
<td>59.8%</td>
<td>50.0%</td>
<td>38.9%</td>
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<tr>
<td>Count</td>
<td>13</td>
<td>41</td>
<td>43</td>
<td>11</td>
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<tr>
<td>% within tumour type</td>
<td>29.5%</td>
<td>40.2%</td>
<td>50.0%</td>
<td>61.1%</td>
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<tr>
<td>Count</td>
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<td>102</td>
<td>86</td>
<td>18</td>
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<tr>
<td>% within tumour type</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
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</tr>
</tbody>
</table>

**Table I.** Relationship of lymph node metastasis with the CXCR4 staining pattern

**Table II.** Distribution of axillary lymph node involvement according to breast cancer subtypes
Additionally, Chu et al. reported that pronounced expression of CXCR4 conferred poor prognosis in basal-like triple-negative breast cancers [19].

Earlier, we reported a correlation between cytoplasmic CXCR4 expression and lymph node metastasis, especially in HER2+ and basal-like triple-negative breast cancer patients, thus supporting the similar observations reported by others as well [20]. Furthermore, Zhang et al. have reported more pronounced expression of CXCR4 in basal-like triple-negative subtype than in Luminal A [21].

Our study also demonstrates the positive relationship between CXCR4 expression and axillary lymph node metastasis, confirming the already established literature findings [22, 23]. Albeit, our study failed to demonstrate any correlation between CXCR4 and breast cancer subtypes, without any detectable difference. Nor was there any correlation with other prognostic variables.

Recently investigations have focused on identifying better prognostic factors to guide clinicians. The significance of TILs in breast tumour microenvironment has been analysed, especially after the discovery of their implications regarding reduced recurrence risk and prolonged overall survival in colorectal and ovarian cancers. In breast cancer, investigators have demonstrated the anti-tumoural activity of cytotoxic T lymphocytes [24]. Denkert et al. demonstrated the favourable prognostic implication of lymphocytic infiltration in patients who received neoadjuvant anthracycline/taxane chemotherapy. These patients attained better outcome after the treatment, contrary to patients whose tumours did not show dense lymphocytic infiltration [2, 3]. Additionally, Loi et al. also demonstrated the prognostic significance of lymphocytic infiltration and suggested the potential favourable role of immunogenic treatments in breast cancer [4]. Likewise, Adams et al. demonstrated the favourable prognostic implication of lymphocytic infiltration in triple-negative breast cancer [5]. Furthermore, the role of tumour-infiltrating lymphocytes as a favourable prognostic marker in early-stage triple-negative breast cancer patients was demonstrated by Ibrahim et al. [25].

Earlier, investigators have demonstrated the positive implication of CD8+ TILs in distant stromal site to have a favourable implication in patients’ survival. In our study, we analysed CD8+ TILs in intratumoural, adjacent stroma, and distant stromal sites as described by Mahmoud et al. [1]. Furthermore, we evaluated the role of CD8+ TILs in different sites with axillary lymph node metastasis; however, we failed to reach any meaningful conclusions. However, Macchetti et al. discovered a statistically significant correlation between CD4+ TILs and axillary lymph node involvement, but they failed to report a meaningful correlation between CD8+ TILs and axillary lymph node involvement [26]. Conversely, Matkowski
et al. found a highly significant correlation between both CD4-positive and CD8-positive TILs and axillary lymph node involvement [27]. Nonetheless, there was a positive correlation between high Ki-67 proliferation index and increased CD8+ TILs in all sites. This was in agreement with the study performed by Khedr et al. [28]. Furthermore, we demonstrated a positive correlation with increasing tumour size and CD8+ TILs infiltration in both intratumoural and adjacent stromal sites, excluding distant stromal sites. We believe dense CD8+ TILs infiltration in patients with increased Ki-67-positive proliferation index and tumour size implies tumour aggressiveness.

We also observed a meaningful correlation with respect to metastatic lymph node involvement between tumour size and lymphovascular invasion. This conclusion was in agreement with the study of Zhang et al. [29].

There has been a statistically significant relationship between breast cancer subtypes and axillary lymph node involvement. In luminal A + B types, the axillary lymph node involvement was less prevalent than in HER2+ breast cancer patients, especially when involvement of more than three lymph nodes was taken into consideration. A similar conclusion was reached by Howland et al. [30].

In our study, 18 out of 250 breast cancer patients were triple negative. Despite reports of strong lymphocytic response and heavy CD8+ TILs infiltration in this subtype, specifically, we did not find any difference when compared to other subtypes. Neither was there any distinctive correlation between CD8+ TILs and the lymph node involvement in this subtype. The lack of such an observation might be related to the small number of triple-negative patients when compared with other subtypes.

Similar to our observation, Seok et al. analysed lymphocytic infiltration in different sites in 133 patients with triple-negative disease and found no correlation with tumour size, lymph node involvement, and tumour grade [31].

In conclusion, CXCR4 staining intensity in breast cancer shows an exponential relationship with axillary lymph node involvement. Similar findings were observed with primary tumour size, HER2+ subtype, lymphovascular invasion, and axillary lymph nodes. There was no correlation between CD8+ TILs and axillary lymph node involvement; nor was there any correlation with lymphovascular invasion and ER, PR expression. However, a reverse correlation was discovered between CD8+ TILs and CXCR4 expression.

These findings may have clinical implications for breast cancer patients, thus providing more insights into the assessment of prognosis as well as redesigning treatment options accordingly, including potential use of anti-CXCR4 antibodies and a new generation of immune modulating agents in the future.

The study was pursued after obtaining formal authorisation by the Institutional Review Board. The identities of all the patients were kept private.

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The authors declare no conflict of interest.

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