Original paper

Lymphoid environment in molecular subtypes of breast cancer

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Recently, a large body of evidence has shown that the microenvironment of invasive breast carcinoma affects its development and the patient's outcome, and vice versa – cancer cells express factors that modulate tumour milieu in terms of its composition and function. We performed an immunohistochemical (IHC) staining of 108 formalin-fixed, paraffin-embedded (FFPE) tissue samples to investigate the relationships between T-cell, B-cell, and NK-cell infiltrate, invasive breast carcinomas molecular subtypes, and other prognostic indicators. The main findings of our study were as follows: the significantly higher infiltrate of the analysed immune cell subsets in triple-negative (TNBC), HER2-positive, non-luminal and luminal B/HER2+ breast carcinomas than in luminal A cancers; their higher densities in poorly differentiated lesions; correlations between lymphoid cells and the expression of hormonal receptors, HER2 receptor status, and marker of cancer proliferation. Furthermore, we observed T-cell numbers to be associated with greater tumour diameter. In summary, the results of our study indicate associations between tumoural lymphoid infiltration and the unfavourable intrinsic subtypes as well as other detrimental prognostic factors in invasive breast carcinomas.

Key words: breast cancer, T-lymphocytes, B-lymphocytes, natural killer cells, tumour microenvironment.

Introduction

Regarding its biology and morphology, breast carcinoma is considered as a heterogenous disease. On the basis of distinct genetic patterns, several molecular subtypes, differing in their clinical behaviour, are distinguished in invasive breast tumours [1, 2]. In routine pathology these subtypes are roughly determined by immunohistochemistry; the stratification of invasive breast tumours into luminal A, luminal B (with or without HER2 overexpression), non-luminal HER2-overexpressing, and triple-negative phenotype significantly influences the patient's treatment and prognosis [1, 3]. Recently, immune cell infiltrate has emerged as a new prognostic biomarker in this malignancy [1, 2].

Inflammatory infiltrate in tumour microenvironment comprises many cell populations that exert a diverse effect on cancer cells, ranging from promoting tumour development to suppressing its growth. The cancer-immune cell interplay results from the direct cell-to-cell contact or is mediated by lymphoid cell-derived molecules, e.g. receptors, cytokines, and chemokines. There is growing evidence that

interactions between neoplastic and immune cells affect the patient's outcome [4, 5, 6, 7, 8, 9]. The cancer milieu includes lymphoid cells, with tumour-infiltrating T- and B-lymphocytes as well as natural killer (NK) cells. These represent both the adaptive and the innate branch of immune response. Concerning T-cells, several subgroups, which differ in their function, phenotype, and cytokine profile, are distinguished. The activity of respective T-cell subsets ranges from cytotoxicity towards malignant cells and anti-tumour response enhancement to immunotolerance induction and immune suppression [4, 6, 10]. Moreover, tumour-infiltrating B-lymphocytes were reported by some authors to be a source of anti-tumour antibodies [11]. Similarly to some T-cells, the propensity of cancer-related NK cells to control tumour growth and its spread is mainly attributed to their cytotoxic activity [12, 13]. Hence, both cellular and humoural immune response are involved in complex tumour-host interactions [14, 15].

Recently, more and more evidence is emerging that tumour-infiltrating cells are substantially affected by both the cancer cells and other elements of the environment. These alterations may involve their composition and function as well as recruitment to the tumour site. This is due to the molecules secreted and expressed by both malignant and stromal cells as well as metabolic alteration within malignant tissue. Nutrient depletion and the accumulation of waste products in the tumour may promote immunity suppression [16, 17, 18, 19]. On the other hand, apoptosis-associated change in the expression of surface and cytoplasmatic tumoural antigens was suggested to enhance immune reaction, a phenomenon observed in highly proliferative tumours and during chemotherapy [10, 14, 20, 21]. Several authors postulated the prognostic value of tumour-infiltrating lymphocyte density and their subpopulations in breast cancer [7, 21, 22], and some even indicated that immune-derived parameters may have stronger prognostic value than tumour-based markers [23]. Thus, the evaluation of tumour-infiltrating lymphocytes (TILs) in breast cancer has recently been proposed as a novel, supplemental indicator of patient outcome due to its possible clinical relevance [7]. In future, more research into immune cell involvement in breast cancer progression may contribute to the development of immunotherapy – a new therapeutic approach aimed at evoking strong effective anti-tumour response as well as at breaking cancer cell escape from immunosurveillance [6, 9, 13, 20, 24, 25, 26].

The aim of our study was to evaluate the densities of tumour-infiltrating T-cells, B-cells, and NK cells in breast cancers of different molecular subtypes, and to investigate their associations with other prognostic markers in this malignancy.

Material and methods

Materials

The materials comprised routinely processed, formalin-fixed, paraffin-embedded tissues of primary invasive breast carcinomas diagnosed between 2002 and 2014. Patients who had received presurgical chemotherapy were excluded from the study. The archival haematoxylin-eosin-stained slides were re-evaluated, and representative, well-preserved specimens were chosen for immunohistochemistry. The Nottingham Histologic Grade system was used for the grading, while the staging was performed according to the 8th edition of the AJCC system [27].

Immunohistochemistry

The specimen processing was largely performed in accordance with the previously described methodology [28]. Immunohistochemistry (IHC) for CD45RO, CD20, CD56, estrogen receptor (ER), progesterone receptor (PR), and Ki67 protein was performed according to the protocol routinely used in our laboratory. The selected blocks were cut into 4-μm-thick sections. Antigen retrieval was performed by incubating the slides in citrate buffer (pH 6.0; 0.01M) or EDTA (pH 8.0; 0.01M) at 97°C in a water bath for 40 and 30 minutes, respectively. UltraVision Quanto detection system (Lab Vision, ThermoScientific, USA) and 3,3'-diaminobenzidine as chromogen were used, and the slides were counterstained with Mayer haematoxylin (Thermo Fisher Scientific, Waltham, USA) and coverslipped. Immunohistochemistry for HER2 (PATHWAY 4B5, Ventana Medical System Inc., USA) was performed automatically on BenchMark BMK Classic autostainer (Ventana, USA) using UltraVIEW DAB Detection Kit (Ventana Medical Systems Inc., USA). The primary antibodies used in the study are listed in Table I.

For specimens with HER2 status 2+ in immunohistochemistry results, fluorescence *in situ* hybridisation (FISH) was conducted. FISH was performed using a PathVysion HER-2 DNA Probe Kit II (Abbott Molecular, USA) according to the manufacturer's protocol. In short, paraffin blocks were cut into 4-μm-thick sections. Hybridisation was performed at 37°C for 14 to 18 hours with a Locus Specific Identifier (LSI) DNA probe (app. 226 kb) SpectrumOrange directly labelled (Abbott Molecular, USA) and a Chromosome Enumeration Probe 17 (CEP17) satellite DNA probe (app. 5.4 kb) SpectrumGreen directly labelled (Abbott Molecular, USA). 4,6-diamino-2-phenylidole (DAPI) was used as a nuclear counterstain. The LSI HER-2/neu and CEP17 signals were counted on a fluorescence microscope equipped with specific filter sets, and HER-2/neu to CEP17 ratio $>$ 2.0 was considered as HER2/neu overexpression [29].

Table I. Antibodies used in the study

	Clone	Dilution	Antigen retrieval	Incubation time	Manufacturer
CD45RO	UCHL1	1 : 100	Citrate	30 min	Dako, USA
CD20	L26	1 : 50	Citrate	30 min	Dako, USA
CD56	MLQ-42	1 : 100	Citrate	30 min	Cell Marque, USA
Estrogen receptor	6F11	1 : 25	Citrate	60 min	Novocastra (Leica Biosystems, Germany)
Progesterone receptor	PgR636	1 : 50	Citrate	60 min	Dako, USA
Ki67	MIB-1	1 : 100	EDTA	60 min	Dako, USA

Evaluation of immunostaining

The slides stained for CD45RO, CD20, and CD56 were initially scanned on a Nikon Labophot-2 optical microscope (Tokyo, Japan) at a very low magnification (25×) to select areas of the highest positive cell infiltrate. For research purposes, three scoring systems were applied to the study:

1. The densities of investigated CD45RO– and CD20-positive cells (T- and B-cells, respectively) were evaluated under a low magnification (100×), as a percentage of tumour tissue area occupied by positively stained cells. The cells located no farther than one 100× power field from the tumour edge were regarded as "invasive margin". Intratumoural population was determined as positively-stained cells located within cancer cell islets or surrounding cancer islets, with direct contact to neoplastic tissue. The cell density was evaluated as an average value scored in 5 power fields of the highest lymphoid infiltrate.

2. The intensity of CD45RO and CD20-positive cell infiltrate was additionally evaluated in accordance with the system of Kreike *et al*. [30]. The grading was as follows: 0 – none, 1 – weak, 2 – moderate, and 3 – intensive lymphoid infiltrate.

3. For evaluation of CD56-positive (NK) cells, the positively-stained cells were first scanned at a low magnification (100×) and the areas with the highest number of positive cells were chosen. Then, positively stained cells were counted in five high-power fields (HPF; 400×, 0.2 mm^2 field area), which represented 1 mm^2 of the examined tissue. The cells located no farther than one HPF from the tumour edge were regarded as "invasive margin". The intratumoural population was determined as positively-stained cells located within cancer cell islets or the positively-stained cells surrounding cancer islets, with direct contact to neoplastic tissue.

Positive ER and PR expression were set when ≥ 1% of neoplastic cells showed positive immunostaining. The threshold for discriminating between low and high Ki67 expression was set at ≥ 20% of positive cells. Scoring of the HER2 staining was performed by standard method [29].

Definition of breast cancer molecular subtypes

The cases were classified into molecular subtypes according to St Gallen 2015 International Expert Consensus [31]: luminal A (ER+ and PR ≥ 20%, Ki67 < 20%, HER2–), luminal B/ HER2– (ER+, HER2– with PR < 20% and/or Ki67 ≥ 20%), luminal B/ HER2+ (ER+ or PR+, HER2+), HER2+ non-luminal (ER–/PR–/HER2+), and triple-negative breast cancer (ER–/PR–/HER2–).

Results

Description of the study group

The study group consisted of 108 primary invasive breast cancer female patients. The average patient age at the time of diagnosis was 55 years (range: 29-87 years). Regarding the stage of the disease, 42 (38.9%) cases were classified as stage I, 41 (38.0%) as stage II, 24 (22.2%) as stage III, and 1 case (0.9%) as stage IV. Tumour sizes were as follows: pT1 – 60 (55.5%), pT2 – 45 (41.7%), and pT3 – 3 cases (2.8%). Concerning lymph node status, 54 (50.0%) patients had no nodal involvement (pN0), while 31 (28.7%) were of stage pN1, 9 (8.3%) of stage pN2, and 13 (12.0%) of stage pN3. Nottingham Histologic Grade distribution was as follows: G1 – 17 (15.7%), G2 – 37 (34.3%), and G3 – 54 (50.0%) cases. With respect to the histologic type, 91 cases (84.3%) were classified as invasive carcinoma not otherwise specified (NOS), 15 (13.9%) cases were of lobular histology (CLI), while for 2 cases (1.8%) the histologic type was determined as "other".

Distribution of molecular subtypes was as follows: luminal A – 36 (33.3%), luminal B – 14 (13.0%), luminal B/HER2+ – 10 (9.3%), non-luminal HER2+ – 20 (18.5%), and triple-negative breast cancer (TNBC) – 28 (25.9%) cases.

Differences in lymphocytic infiltrate between respective breast cancer molecular subtypes

First, the differences in tumour area occupied by T-cell, B-cell, and NK cell infiltrate were investigated between tumours of either luminal or non-luminal

Table II. T-cell, B-cell, and NK cell densities with reference to breast cancer molecular subtypes and HER2 status; the values were measured as a percentage of tumour area occupied by positively-stained immune cells

	T-cells				B-cells				NK cells			
	Intratumoural		Invasive margin		Intratumoural		Invasive margin		Intratumoural		Invasive margin	
	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p
Molecular subtype												
Luminal A	3.29 (3.15)	< 0.015	13.31 (11.24)	< 0.001	1.12 (1.71)	< 0.03	9.59 (10.96)	< 0.002	5.46 (5.10)	NS	13.03 (10.24)	< 0.01
Luminal B	4.75 (3.93)		20.93 (14.06)		1.33 (2.37)		10.43 (5.76)		5.50 (6.53)		12.46 (8.63)	
Luminal B/ HER2+	8.00 (8.77)		40.56 (24.77)		2.65 (3.94)		21.70 (21.16)		7.10 (6.15)		16.40 (11.22)	
HER2+ non-luminal	6.53 (6.28)		37.67 (23.51)		3.48 (4.70)		20.79 (13.28)		14.25 (19.48)		29.21 (42.00)	
TNBC	10.24 (10.50)		30.96 (19.40)		3.41 (4.57)		16.52 (12.12)		14.96 (29.42)		31.46 (22.34)	
Molecular phenotype												
Luminal	4.38 (4.86)	< 0.008	19.38 (17.34)	< 0.001	1.44 (2.40)	< 0.002	11.88 (12.97)	< 0.001	5.75 (5.54)	NS	13.47 (10.00)	< 0.001
Non-luminal	8.70 (9.10)		33.59 (21.11)		3.44 (4.57)		18.28 (12.65)		14.65 (25.32)		30.51 (31.72)	
HER2+ status												
Normal	6.10 (7.56)	NS	21.12 (16.96)	< 0.001	2.00 (3.31)	NS	12.24 (11.02)	< 0.006	8.85 (18.44)	NS	19.32 (17.59)	NS
Overexpressed	7.00 (7.04)		38.63 (23.50)		3.19 (4.39)		21.10 (16.04)		11.87 (16.50)		24.79 (34.83)	
Ki67 status												
low	4.16 (4.50)	< 0.015	16.34 (15.92)	< 0.001	1.56 (2.77)	< 0.04	10.72 (10.57)	< 0.002	5.49 (5.38)	NS	13.52 (11.78)	< 0.001
high	8.02 (8.71)		33.63 (20.45)		2.95 (4.14)		17.88 (14.20)		13.00 (23.10)		27.13 (28.98)	

NS – not significant

phenotype. The significantly higher densities of T- and B-lymphocytes, both in intratumoural area and at the invasive margin, were observed in non-luminal tumours, as compared with luminal ones. A similar observation was made for NK cells at the tumour edge (Table II). Then, the evaluation with reference to breast cancer molecular subtypes was performed (in accordance with St Gallen 2015 recommendations). The density of intratumoural T-cells was significantly higher in TNBC than in luminal A tumours ($p < 0.015$). Likewise, luminal A subtype was associated with lower T-lymphocyte densities at the invasion front in comparison with TNBC ($p < 0.001$), HER2+ non-luminal ($p < 0.001$), and luminal B/HER2+ ($p < 0.005$) tumours. Statistically significant differences in intratumoural B-cell infiltrate between respective subtypes were observed in Kruskal-Wallis ANOVA test exclusively. For this subpopulation, the cell density increased from luminal A and B tumours to HER2+ non-luminal and TNBC cancers. B-cell infiltrate at the invasive edge was more abundant in TNBC and HER2+ non-luminal tumours as compared to luminal A lesions ($p < 0.03$ and $p < 0.002$, respectively). With reference to NK cells, the only statistically significant differences were observed at the tumour invasive margin. The cell densities were significantly lower in luminal A and B in comparison with TNBC cancers ($p < 0.001$ and $p < 0.3$, respectively; Table II, Fig. 1A).

As far as the luminal subtypes were concerned, increased T-cell infiltrate of luminal B/HER2+ invasive margin, in comparison with luminal A cancers, were the only statistically significant differences observed, according to St Gallen either 2015 or 2013 classification (Ki67 expression cut-off ≥ 20% or ≥ 14%, respectively; $p < 0.003$). However, the infiltrates of all investigated subsets were highest in luminal B/HER2+ tumours. None of the analysed immune cell subpopulations differed significantly in their quantities between luminal A and B cancers, regardless of the St Gallen classification applied.

The evaluation of T- and B-cell infiltrate intensiveness, performed according to the system of Kreike *et al*., was partially concordant with the above-mentioned results. Statistically significant differences between groups were observed for T-lymphocytes at the invasive margin ($p < 0.006$), as well as for B-cells, both within the tumour bed and at the invasive front ($p < 0.035$ and $p < 0.03$, respectively). Once again, the immune cells were less abundant in luminal A and B cancers compared to HER2+ non-luminal and TNBC lesions. The differences, however, did not reach statistical significance in post-hoc test (Fig. 1B).

All analysed immune cell populations, excluding intratumoural NK cells, showed slight to moderate negative correlations with both the ER and the PR expression, as well as positive correlations with the expression of Ki67 and mitotic index. However, the only immune cell populations that displayed correlation with HER2 expression were T- and B-cells located at the invasive margin (data not shown). Almost all of the investigated immune cell subsets were increased in highly proliferating tumours (Ki67 ≥ 20%), with the exception of intratumoural NK cells (Table II). Similar results were obtained with the system of Kreike *et al*. for T-cells and B-cells at the invasion front exclusively. The increased numbers of T- and B-lymphocytes at the invasion edge were also observed in HER2-overexpressing tumours, both when evaluated as a percentage of the tumour area involved or according to the system of Kreike *et al*. (Table II).

Associations between lymphocytic infiltrate and other prognostic indicators in breast cancer

For all the analysed immune cell populations, the percentage of the tumour area infiltrated by immune cells differed significantly between lesions of respective Nottingham Histologic Grade. The infiltrates of either T- or B-cells, both within tumour nest and at the invasive margin, as well as of NK cells at the invasion front, were significantly lower in G1 and G2 than in G3 cancers (the highest p value < 0.01). Regarding intratumoural NK cells, the differences were only found between G2 and G3 cancers (Table III, Fig. 2A). Similar results were obtained with the scoring system of Kreike *et al*. for T-cells (both in intratumoural area and at the tumour edge) as well as for intratumoural B-cells ($p < 0.001$). With reference to B-lymphocytes of the invasive margin, the statistical significance between groups was reached in Kruskal-Wallis ANOVA exclusively ($p < 0.04$, Fig. 2B).

Regarding pTNM staging, we found T- and B-cell infiltrates of the invasion front to be significantly less abundant in stage I cases in comparison with stage II patients ($p < 0.04$, Table III). No statistically significant associations with the stage of the disease were obtained with the system of Kreike *et al.*

A higher percentage of the tumour area infiltrated by T-cells was observed in cancers of diameter larger than 2 cm (pT > 1) in comparison with pT1 tumours (tumour bed population – $p < 0.02$, invasion margin – $p < 0.03$, Table III). When the system of Kreike *et al*. was applied, only the higher intratumoural T-cell infiltrate density was significantly associated with greater tumour size. Regardless of the evaluation system applied, no differences in lymphocytic densities were found between tumours with various lymph node status (data not shown).

Regarding the histologic type, a higher number of T- and B-cells at the invasive margin was

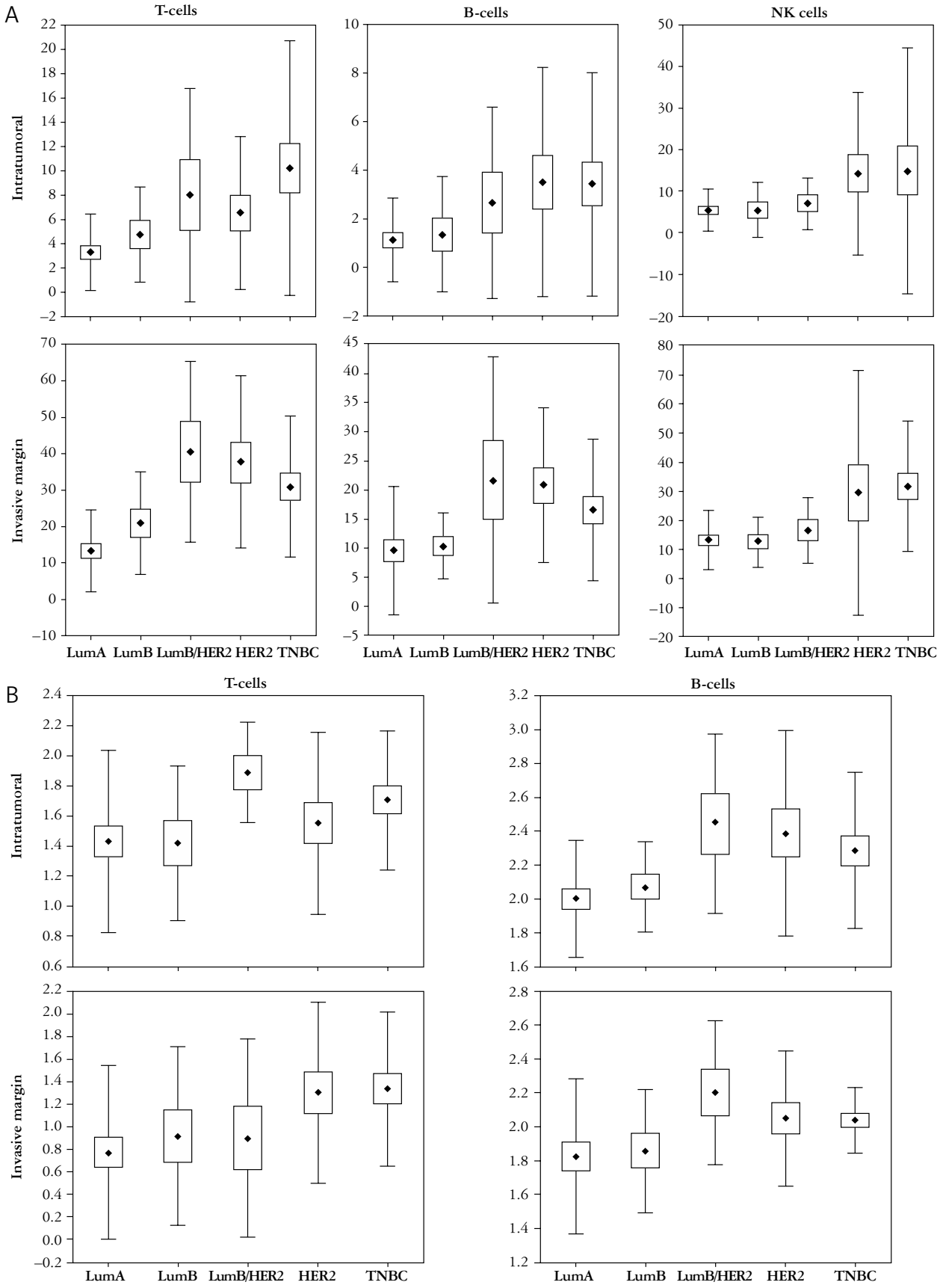

Fig. 1. The densities of T-cells, B-cells, and NK cells with reference to St Gallen 2015 molecular subtype. A) Immune cell quantities evaluated as a percentage of tumour area involved; B) Infiltration density assessed by the scoring system of Kreike *et al*. Central point is the arithmetic mean, box is the arithmetic mean ± standard error, and whisker is the arithmetic mean ± standard deviation. ANOVA Kruskal-Wallis test

Table III. T-cell, B-cell, and NK cell densities in breast cancers of different stage, tumour size, grade, and histologic type; the values were measured as a percentage of tumour area occupied by positively-stained immune cells

	T-cells				B-cells				NK cells			
	Intratumoural		Invasive margin		Intratumoural		Invasive margin		Intratumoural		Invasive margin	
	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p	Mean (SD)	p
Stage												
I	4.75 (7.56)	< 0.045	17.95 (14.59)	< 0.015	1.80 (3.35)	NS	10.49 (10.20)	< 0.02	11.41 (25.79)	NS	21.03 (30.72)	NS
II	8.06 (8.06)		30.24 (22.14)		2.92 (4.34)		17.85 (15.36)		8.38 (10.72)		20.46 (18.95)	
III	6.32 (5.43)		31.95 (21.84)		2.30 (2.82)		16.57 (12.10)		9.61 (10.80)		22.00 (16.93)	
Tumour size												
pT1	5.65 (8.08)	< 0.02	21.79 (18.56)	< 0.03	2.25 (4.01)	NS	13.57 (13.02)	NS	11.46 (22.09)	NS	20.84 (28.82)	NS
pT < 1	7.22 (6.42)		30.36 (21.45)		2.43 (3.22)		16.04 (13.33)		7.59 (10.41)		20.85 (15.46)	
Nottingham Histologic Grade												
G1	2.19 (2.03)	< 0.001	11.81 (7.51)	< 0.001	0.31 (0.57)	< 0.001	8.18 (5.76)	< 0.001	5.18 (5.29)	< 0.02	13.41 (12.44)	< 0.001
G2	3.14 (2.88)		16.69 (14.39)		1.00 (1.21)		11.31 (12.79)		4.58 (4.91)		14.57 (13.30)	
G3	9.67 (8.80)		36.13 (21.05)		3.80 (4.54)		19.13 (13.72)		14.40 (23.61)		27.50 (29.50)	
Histologic type												
NOS	6.77 (7.80)	NS	28.28 (20.72)	< 0.001	2.54 (3.90)	NS	16.09 (13.62)	< 0.001	10.07 (19.23)	NS	21.99 (25.09)	NS
CLI	3.72 (3.55)		9.21 (5.87)		1.15 (1.03)		5.50 (3.78)		7.00 (5.03)		13.36 (10.70)	

NS – not significant

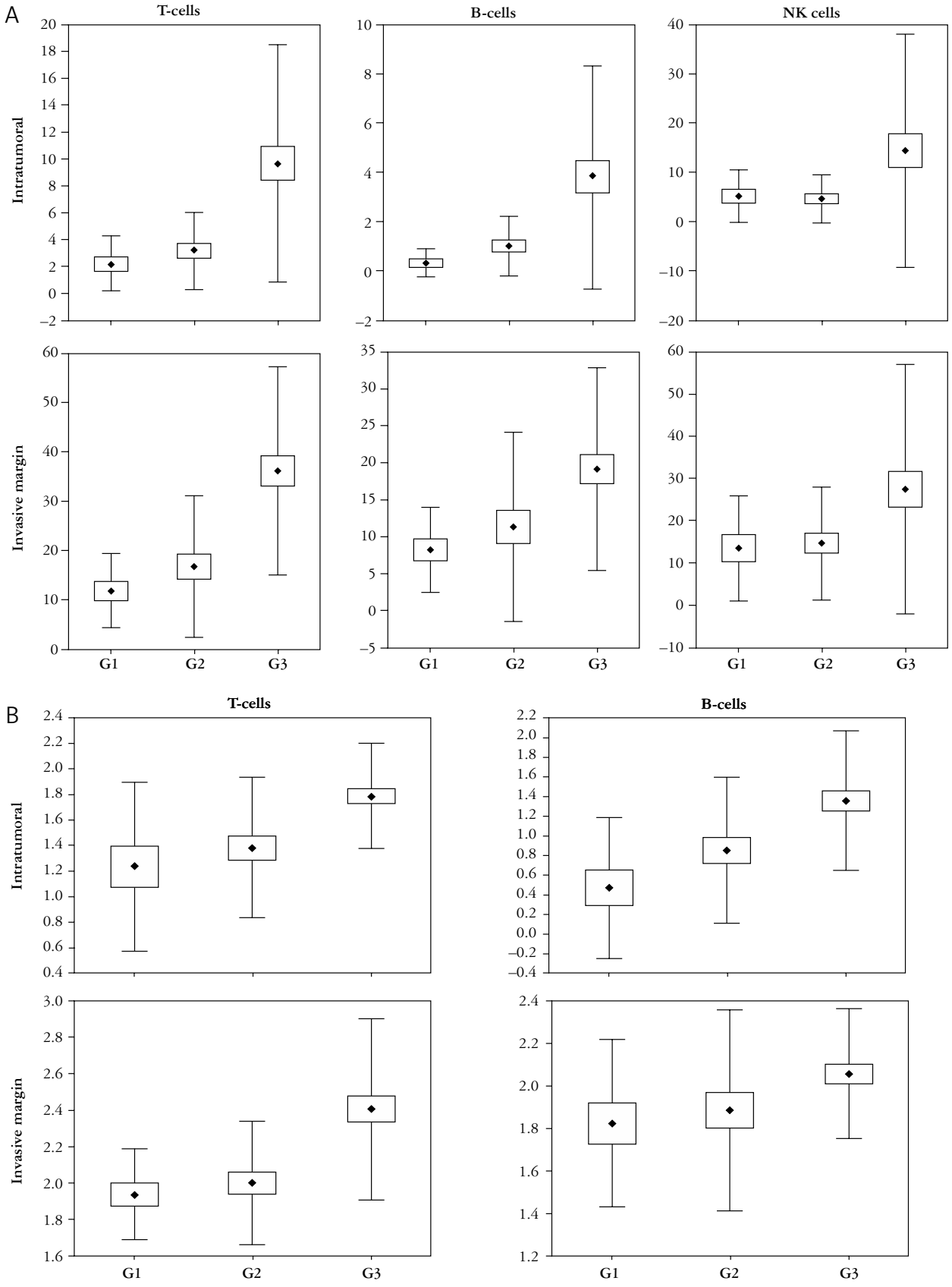

Fig. 2. The densities of T-cells, B-cells, and NK cells with reference to Nottingham Histologic Grade. A) Immune cell quantities evaluated as a percentage of tumour area involved; B) Infiltration density assessed by the scoring system of Kreike *et al*. Central point is the arithmetic mean, box is the arithmetic mean ± standard error, and whisker is the arithmetic mean ± standard deviation. ANOVA Kruskal-Wallis test

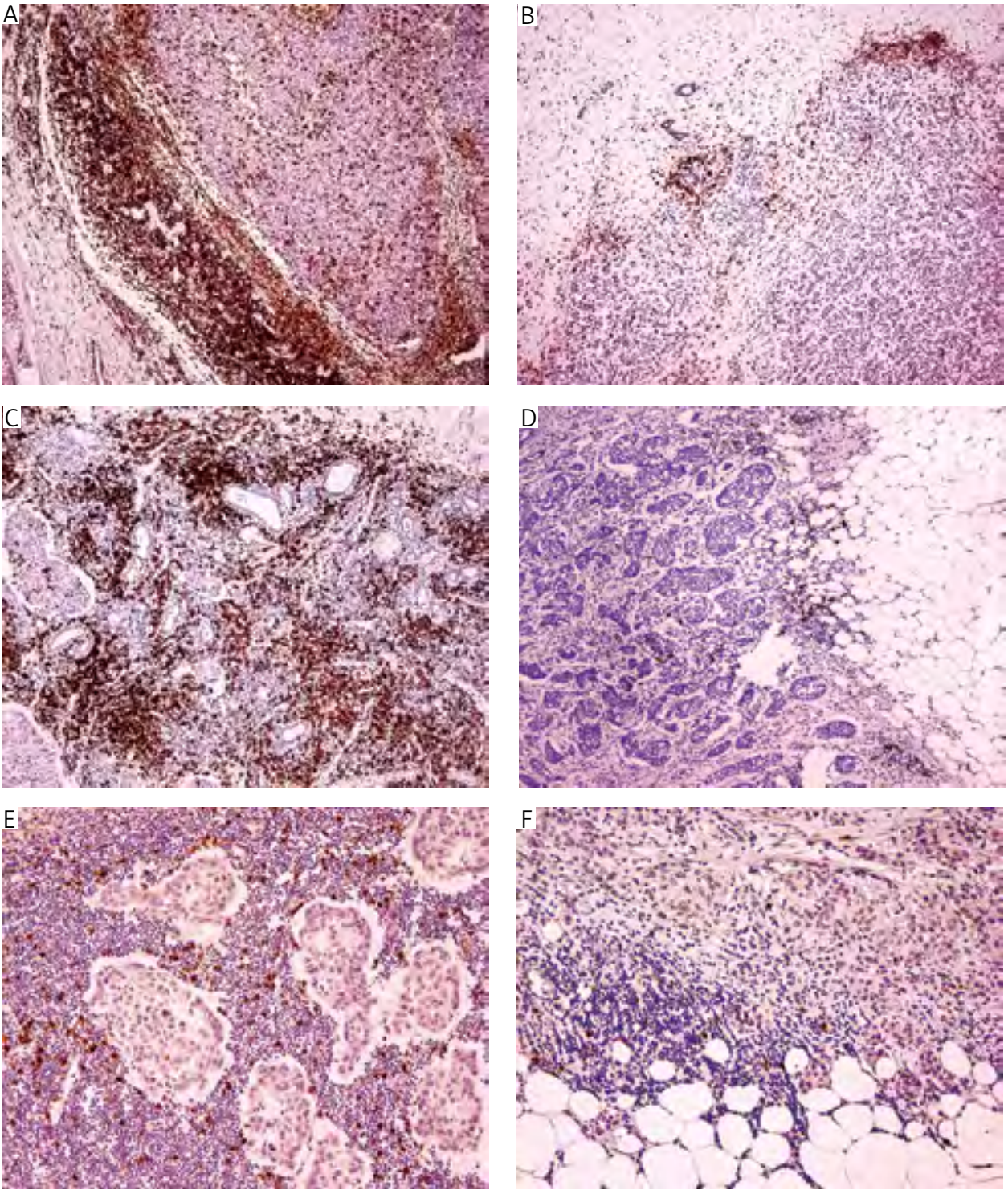

Fig. 3. Lymphoid infiltration of invasive breast cancer tissue. Abundant and low densities of T-cells (A, B), B-cells (C, D), and NK cells (E, F). Immunohistochemical staining for CD45RO, CD20, and CD56, light microscopy, magnification used: 50× (A-D) and 100× (E, F).

observed in NOS cancers, as compared to CLI lesions (Table III). This observation was significant either when the percentage of the infiltrated tumour area or the system of Kreike *et al*. was concerned (data not shown).

Discussion

Although determining the molecular subtypes has become standard in breast cancer management, the information concerning the relationship between the molecular and immune phenotype of the tumour

is scarce and, to a certain extent, inconclusive. More abundant lymphocyte infiltrate observed by some authors in non-luminal breast cancer tumours, as compared to the luminal ones, was suggested to be associated with their more aggressive profile, genetic instability, HLA-G expression, and distinct metabolism [21, 24, 32, 33]. Moreover, intense immune infiltration as well as higher cytokine level were reported in high-grade, hormone receptor-negative [8, 23, 34, 35], and HER-2 overexpressing breast tumours [8, 34, 35]. To date, it was shown that the clinicopathological significance of TILs in breast cancer is phenotype-dependent and ranges from their anti-tumour to pro-tumourogenic properties because the more prominent lymphocytic infiltrate was associated with either more beneficial prognosis in TNBC [24] and ER–/HER2+ [21, 34] lesions or with an unfavourable patient outcome in ER+ cancers [34]. Similarly, Nagalla *et al*. observed that the immune gene expression was either a beneficial indicator of distant metastasis-free survival in highly and intermediately proliferative or an adverse factor in low proliferative cancers [36]. This was also supported by other studies, which pointed out that the impact of immune metagene on the prognosis depended on both the molecular subtype and the proliferation status of breast tumour [36, 37].

Out of all the TILs, T-cells are considered as a prevailing subpopulation [4, 6, 8, 38, 39]. In our study more abundant T-cell infiltrate was associated with more aggressive breast cancer molecular subtypes: TNBC, HER2+ non-luminal, and luminal B/HER2+. Moreover, differences between luminal A and TNBC lesions concerned both the tumour nest and its immediate surrounding. A similar observation was made by Cimino-Matthews *et al*., who described a higher T-cell infiltration in human primary TNBC as compared with luminal tumours [38]. Some authors postulated associations between tumour infiltrating T-cell quantity and ER- as well as PR-negativity [40], which is in accordance with our results, while others did not [41]. Research into microinvasive breast cancer provided a hypothesis on immunogenicity of HER2-overexpressing tumours, which leads to the accumulation of cytotoxic T-cells, and, finally, to the rupture of the basement membrane [42]. Moreover, among luminal lesions, we noted that T-cells located at the invasive front of malignant lesions were the only subpopulation that differed significantly, with their highest density in luminal B/HER2+ lesions. Thus, we hypothesise that HER2 overexpression influences T-lymphocyte response (or vice versa) to a greater extent than is achieved by the higher proliferation and the decrease in hormone receptors (two factors that discriminate between luminal A and B cancers). Our observation of T-cells being more numerous in high-grade breast tumours than in more differentiated lesions is corroborated by results from other study groups [39, 43]. Two explanations for this phenomenon are offered: the T-cell contribution to the cancer aggressiveness or the impact of high-grade tumours on immune response [38, 41]. We found that T-lymphocytes were the only population significantly increased in tumours of greater diameter. On the other hand, some authors indicated smaller tumour diameter, lower grade, fewer positive lymph nodes, and longer survival as characteristic of invasive breast ductal carcinomas abounding in T-cells [44]. These were suggested to control tumour progression due to their ability to eliminate cancer cells and to prevent metastasis formation [44, 45]. Such a hypothesis was supported by a study in a murine model, in which tumour-specific T-cells were noticed in bone marrow. After stimulation the cells penetrated the malignant breast tumour and reduced its size [46]. Interestingly, along with an increase in the histologic grade of an early breast cancer, a shift from a naive towards a memory and an activated T-cell phenotype was observed [47].

The crosstalk between T-cells and breast cancer is complex. Fu *et al*. proposed a hypothesis of T-cells influencing fibroblast function, which in turn may promote tumour progression. In addition, the authors observed a high ratio of regulatory T-lymphocyte subpopulation within breast cancer tissue [16]. On the other hand, an increased percentage of cytotoxic T-cells in primary breast tumour site may indicate a favourable prognosis [23]. Our study showed that more abundant T-cell densities were associated with adverse pathological prognostic factors, such as greater tumour size, HER2-overexpression, and higher proliferation rate. Interestingly, the increased T-cell density in breast tumour stroma and within tumour nest was reported to correlate with longer survival [41]. Moreover, a higher prevalence of T-cell than B-cell fraction was proposed as the indicator of pathological complete response (pCR) after chemotherapy [8].

To date, an exploration of B-cell densities with reference to breast cancer intrinsic subtype was undertaken only by a few research groups. In a study by Mahmoud *et al*. on invasive ductal carcinomas, high B-cell infiltrate correlated with the lack of ER and PR expression and basal-like phenotype, either in distant or adjacent stroma as well as intratumourally [15]. This, in general, is in accordance with our study, although we found that the correlations between B-cells and hormone receptor status are not strong. On the other hand, more numerous total B-cells in tumour tissue resulted in a favourable outcome, particularly in high-grade, ER-negative, HER2-overexpressing, and basal-like carcinomas [15]. The diverse infiltrate is presumed to persist and even deepen as cancer spreads, with decreased B-cell densities in

TNBC secondary tumours as compared to luminal metastases and primary lesions [38]. More numerous B-lymphocytes were associated with positive HER2 status in primary invasive [40] and high-grade ductal cancers [15, 23]. In line with these findings, we observed B-cells to be increased in non-luminal (HER2-overexpressing and TNBC), HER2-overexpressed, highly proliferating, and poorly differentiated breast tumours. With the exception of the last two groups, the differences were more pronounced at the tumour edge; however, regarding the intrinsic subtype, luminal A and B cancers were less infiltrated also intratumourally. Interestingly, one study observed significantly higher B-cell quantities in G3 within the breast tumour area exclusively [48], and some authors did not observe any associations with cancer differentiation [39]. Similar to the T subset, the average number of B-lymphocytes was higher in luminal B/HER2+ than in the remaining luminal tumours; however, the relationship did not reach statistical significance. Although luminal A and B cancers appear to be closely related, these tumours are distinct entities, with a diverse genetic alteration pattern [49], which do not seem sufficient to translate to varied immune response.

In HER2-negative invasive ductal breast lesions, B-cell proliferation and affinity maturation were suggested to occur at the tumour site and to be antigen-driven [20, 50, 51]. This supported the hypothesis of spontaneous specific humoural response to neoplastic cells [20, 50]. In medullary breast carcinoma, cancer-associated B-cells are a source of antibodies binding to β-actin, which is expressed on the surface of cancer cells during apoptosis [11]. It is thought that the interplay between T- and B-cells results in their mutual stimulation and, consequently, in enhanced immunosurveillance [52]. Conversely, the development of the regulatory subset of B-lymphocytes, in the presence of mammary adenocarcinoma cells, was observed in a murine model; these, in turn, mediate T-cell conversion into a regulatory subset [53]. Some authors postulate that tumoural lymphocytes display the ability to express metalloproteinases, which mediates the recruitment of other immune cells, and consequently promotes cancer progression [17]. Thus, the impact of the microenvironmental B-cells on breast cancer may be twofold, with their pro-tumourogenic activity on the one hand and the enhancement of anti-tumour properties on the other. Moreover, the existence of multiple B-lymphocyte subsets that differ with respect to their phenotype and function is postulated by some authors [25].

The published literature on the relationships between the quantities of NK cells and the molecular subtype of breast cancer is scarce, and so far no significant differences have been shown. However, Engels *et al*. postulated an increased amount of NK cells as one of the positive prognostic factors in luminal A cancers [54]. Moreover, pathological response of HER2-overexpressing tumours to trastuzumab therapy is partially dependent on the enhanced activation of NK cells in reaction to the antibody [13, 21, 22]. We found NK cell infiltrate to be less abundant in luminal as compared with TNBC tumours, but the significance was noted only with reference to the invasive margin population of these cells. Because no significant differences were observed in the NK cell densities between luminal tumours, with their slight increase in luminal HER2-enhanced cancers, more numerous NK cells at the invasive edge of high-proliferating tumours may be explained by higher densities of this population in non-luminal lesions. The higher intratum oral tumoural NK cell infiltration observed in NOS breast cancers was related to higher grade, greater tumour size, and nodal involvement [48]. In contrast, in our study high histologic grade was associated with more numerous NK cells at the invasive margin and no relationship with tumour diameter was obtained. Furthermore, some authors noted a lack of NK cells within neoplastic lesions of the breast [55], which is discordant with our study.

NK cells were proposed as part of an important barrier against metastasising in invasive breast tumours [56]. The expression of NK cell activation and signalling-associated markers, as well as NK cell interactions with dendritic cells and macrophages, were related to longer overall and recurrence-free survival in breast cancer patients in the study by Ascierto *et al*. [12]. In contrast, the activity of genes for CD56, which was considered by some authors as a marker of immature NK cell subgroup [57], did not exhibit any relationship with cancer progression [12]. It is worth noting that the NK cell function is highly dependent on tumour-derived molecules [12, 13], and the existence of several distinct NK cell subsets was postulated by some authors [58]. In certain studies, their cytotoxicity was suggested to increase in the presence of mammary cancer cells [58], while other studies pointed out that breast cancer cells may considerably inhibit the NK cell cytolytic function, and thus promote immune escape [57]. Several explanations were proposed for this phenomenon: an alteration in the expression of receptors and their ligands on NK cells with their shift towards the inhibitory phenotype; an increased expression of cancer-derived molecules, which negatively influences NK cell activity; and a blockade in their terminal maturation at the tumour site [57].

In summary, our results point out the relationships between lymphoid infiltrate and adverse clinicopathological factors in invasive breast cancer, particularly with its less favourable molecular subtypes. Moreover, the differences obtained in our study varied

with reference to immune cell subsets and their location within tumour tissue. The latter observation was most apparent for the NK cells, whose number differed significantly only at the invasive margin, when breast cancer intrinsic subtype or proliferation status were concerned. This might be due to the poor immune cell penetration or the intratumoural population being influenced more by cancer-derived molecules. Considering their "native" properties, either T, B, or NK cell functions appear to be altered within the cancer microenvironment, both intratumourally and in tumour stroma. It is suggested that changes in quantities of the respective immune cell populations may reflect an increase in total TILs [7]. Our previous study on mast cells, however, challenges these results because higher mast cell numbers were observed in luminal breast cancers [28], which are usually regarded as less infiltrated tumours [8, 21, 24]. Thus, further research concerning the tumour microenvironment is needed to elucidate its complex relationships with breast cancer molecular subtypes.

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