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From Cell Biology to Tissue Engineering

The evaluation of the distribution of CD133, CXCR1 and the tumor associated macrophages in different molecular subtypes of breast cancer

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Summary. Breast cancer has different molecular subtypes, which determine the prognosis and response to the treatment. CD133 is a marker for cancer stem cells in tumor microenvironment with diagnostic/therapeutic importance. The tumor associated macrophages (TAMs) interact with the cancer stem cells through the CXCR1 receptor. In this study, we wanted to investigate the expression of these markers in patients with different molecular subtypes, in order to detect pathophysiological mechanisms and new molecular targets for the prospective targeted therapies. In this study we hypothesized a difference in expression of these antigens among different subtypes. We investigated expression of antigens in breast cancer patients with luminal A (LA), luminal B (LB), HER2 overexpressing (HER2OE), triple negative (TN) subtypes (n=70) and control patients (n=10) without cancer diagnosis. We applied indirect immunohistochemistry and evaluated immunostaining. CD133 expression was at the periphery and CXCR1 expression was at the central area of the tumor. The cytoplasmic CXCR1, CD133 expressions and nuclear CD133 expression, which is prominent in the TN subtype, were observed in patients. There was a statistically significant difference between the groups for CD133 (p=0.004), CXCR1 (p=0.002) H-Score values and M2 macrophages/whole TAM ratios (p=0.022). Between the CD133 and CXCR1 H-scores, there was a weak positive correlation (r=0.249, p=0.035). This study showed the compartment specific expression of the CD133 and CXCR1 antigens in neoplastic cells. The use of CD133 as a stem cell marker may be limited to TN subtype, due to its heterogeneous expression.

Key words: Breast Neoplasms, Neoplastic Stem Cells, Interleukin-8A, Macrophages/pathology, Tumor Microenvironment

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Introduction

Breast cancer remains one of the main sources of morbidity and mortality in female cancers. Worldwide, 1.67 million patients were diagnosed with breast cancer, and the number of deaths caused by this malignity was 522,000 in 2012 (Ferlay et al., 2015).

Breast cancer has different molecular subtypes which determine prognosis and treatment response. Breast cancer can be classified into luminal A (LA), luminal B (LB), HER2 overexpressing (HER2OE) and triple negative (TN) subtypes, according to the expression status of estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) receptors and the Ki67 index (Schnitt, 2010). Tumors are heterogeneous structures composed of neoplastic cells, vessels, immune system cells and stromal components (Gomes et al., 2016).

Breast cancer stem cells (BCSC) affect invasion, metastasis and drug resistance, and they are identified with the surface markers of CD44, CD24, ALDH1,

CD133 and CD49f (Aomatsu et al., 2012). Molecular subtypes are also diverse in the expression of the cancer stem cell markers. While the TN subtype is abundant in $CD44^+/CD24^-$ BCSC phenotype, $ALDH1^+$ phenotype predominates in HER2OE subtype (Schmitt et al., 2012).

Tumor associated macrophages (TAMs) are abundant in tumors. The surface markers for TAMs are the pan-macrophage marker CD68 and CD163 for the macrophages with M2 phenotype (Tang, 2013).The monocytes or the resident macrophages can polarize into anti-tumorigenic M1 or pro-tumorigenic M2 macrophages according to their microenvironment . The TAMs interact with the BCSC (Mantovani and Locati, 2013).

The cells of the tumor, mesenchymal stem cells and macrophages in tumor microenvironment secrete IL-8, which binds to the C-X-C motif chemokine receptor 1 (CXCR1) and CXCR2 receptors of the cancer cells, and favors the cancer stem cell phenotype in cooperation with the HER2 receptor (Singh et al., 2013).

The distributions of CD133, CXCR1 and the fraction of CD163 expressing M2 macrophages in the LA, LB, HER2OE and TN subtypes of breast cancer have not been shown previously in patient specimens. In this study, we wanted to investigate the expression of these markers in patients with different molecular subtypes. We consider that detection of these molecular distribution patterns may contribute to the knowledge on pathophysiological mechanisms and new molecular targets for the prospective targeted therapies. In this study we hypothesized a potential difference in expressions of these antigens among different subtypes.

Materials and methods

Patient selection

The participants were selected randomly from the patients, who were diagnosed as breast cancer between 1999 and 2012 (with a median diagnosis year of 2008 and interquartile range of 6 years) in a regional research and training hospital. Patients with sufficient specimens in pathology archives and digital recordings on hospital automation systems were included to the study. The patients who lacked sufficient data for the determination of molecular subtype (including ER, PR, HER2 and Ki67 status) were excluded from the study. The molecular subtypes were determined in n=70 breast cancer patients. While the majority of patients (n=36) were LB subtype, 22 patients had LA and 7 patients had TN subtype. Only 5 patients had HER2 overexpressing subtype.

Ethical approval

This study was approved by Hacettepe University Non-interventional Clinical Research Ethics Board in 03.18.2015 (GO15/174-05).

Tissue microarray

Paraffin blocks (n=7), which contain 27 or 28 patient samples with primary tumor, were prepared and consequently 190 paraffin sections were evaluated. Each patient was evaluated in multiple sections on different blocks, which allowed us to assess antigenic expressions more accurately and increased the reliability of the measurement. In addition, 10 breast tissue samples from the patients without cancer diagnosis were included in the study as the control group.

Immunohistochemistry

Sections with a thickness 3-4 μm were cut with a Leica SM2010R sliding microtome. Antigen retrieval was achieved with a pressure cooker and citrate buffer (pH 6.0 with 0.05% Tween20) for CXCR1 and CD163, or alternatively with microwave oven in 300W and citrate buffer (pH 6.1) for CD133. Rabbit polyclonal IgG anti-CXCR1 (#ab124344, Abcam, Cambridge, MA) and anti-CD163 (#ab87099Abcam, Cambridge, MA) antibodies were applied in 1:200 and 1:300 dilutions, respectively. Mouse monoclonal IgG anti CD133 antibody (#130-090-422, Miltenyi Biotec, Germany) was applied in 1:10 dilution. Goat polyclonal IgG anti-rabbit antibody (#ab6721, Abcam, Cambridge, MA) and goat polyclonal IgG anti-mouse antibody (#ab6789, Abcam, Cambridge, MA) were applied on sections in 1:1000 and 1:500 dilutions, respectively. DAB (3,3'-Diaminobenzidine) and Mayer's hematoxylin were used before mounting.

For the immunostaining of the ER, PR, HER2 and MIB-1 (for Ki67 index), heat mediated antigen retrieval (100°C) was performed. Peroxide Block for Image Analysis (ScyTek Laboratories, West Logan, UT) and Super Block (ScyTek Laboratories, West Logan, UT) were applied for 10 and 5 minutes, respectively. Primary antibodies, anti-ER (#6F11, Leica Biosystems, Germany), PR (#PGR-312, Leica Biosystems, Germany), HER2 (#CB 11, Leica Biosystems, Germany) and MIB-1 (#IR626/IS626, Dako, Glostrup, Denmark) were applied for 20 minutes with dilutions 1:40, 1:150, 1:40 and 1:150, respectively. CRF Anti-Polyvalent HRP Polymer (ScyTek Laboratories, West Logan, UT) was applied for 30 minutes. DAB solution and Mayer's hematoxylin were used before mounting.

Imaging of the specimens

The sections were imaged with the Olympus BH2 and Leica DM6000B upright light microscopes. Micrographs were recorded with Leica DC490 digital camera and LAS v3.8 digital software. For each immunostaining, at least 5 areas were evaluated with x40 objective magnification.

The Scoring of the Immunohistochemistry

For the evaluation of CXCR1 and CD133

immunostaining, H-Score was used, which is dependent on both intensity (0,+1,+2 or +3) and percentage (from 0% to 100%) of membrane staining (Tan et al., 2016). This score ranged between 0 and 300 and was calculated using the formula below:

H-Score = (3 x percentage of the cells with high level staining (+3) intensity) + (2 x percentage of the cells with moderate level staining (+2) intensity) + (1x percentage of the cells with weak level staining (+1) intensity)

For evaluation of CD163 immunostaining, the percentage of $CD163^{+}$ macrophages to whole macrophage population (M2/Total Macrophages) was calculated.

Statistical analysis

Before performing group comparisons and correlation tests, normality distribution assumption was checked with Shapiro-Wilk's test. Mann Whitney U test was applied to compare two independent groups. Kruskal-Wallis test was utilized to compare more than two independent groups. Dunn-Sidak test was used to perform post-hoc tests. Spearman's rho coefficient was used to investigate correlation between numerical variables. Pearson Chi-square test was performed for analyzing relationships between categorical variables. A significance level of 0.05 was used for all statistical tests. As descriptive statistics, median and inter-quartile range (IQR) are presented for numerical variables, frequencies and proportions are presented for categorical variables. IBM SPSS version 19.0 was used to perform statistical analyses. Stata 15.1 was used for visualization of data.

Results

Demographic statistics

There was no significant difference among the groups in terms of patients' ages (p=0.235). Although it is not statistically significant, LA patients were the oldest (median=64.50, IQR=18.50) and LB patients were the youngest patients (median=52.00, IQR=15.50). The majority of the patients were female (n=77, 96.3%), but 3 male patients were spread over LA, LB and TN groups (Table 1).

Histopathological evaluation

The molecular subtypes showed nonspecific findings, like tubular structures or cellular cords (Figs. 1-4). The density of the infiltrating cells in non-luminal subtypes was higher than luminal subtypes (Fig. 2a-2d). The majority of the infiltrating cells were classified as macrophages, according to morphological features and their expression of CD163 antigen. CD133 expression was at the periphery of the tumor clusters (Fig. 1a) and CXCR1 expression was at the central areas (Fig. 3a-3b). Cytoplasmic CXCR1, CD133 expressions and nuclear

Table 1. The demographic features and ER, PR, HER2 receptor expression, Ki67 index statuses of the patients.

			LA	LB	HER2OE	TN	Control	Total	p-Value
N			22	36	5	7	10	80	N/A
Frequency in %			27.5	45.0	6.3	8.8	12.5	100	N/A
Age	Median		64.50	52.00	53.00	53.00	58.00	N/A	p=0.235
	Percentiles	25	50.75	48.25	48.00	48.00	39.50		
		75	69.25	63.75	64.00	64.00	60.25		
Sex	Female	N	21	35	5	6	10	77	N/A
		%+	95.5	97.2	100.0	85.7	100.0	96.3	
	Male	N	1	1	0	1	0	3	
		%+	4.5	2.8	0.0	14.3	0.0	3.8	
ER	Negative	N	1	3	5	7	N/A	16	N/A
		%+	4.5	8.3	100.0	100.0	N/A	22.9	
	Positive	N	21	33	0	0	N/A	54	
		%+	95.5	91.7	0.0	0.0	N/A	77.1	
PR	Negative	N	2	8	5	7	N/A	22	N/A
		%+	9.1	22.2	100.0	100.0	N/A	31.4	
	Positive	N	20	28	0	0	N/A	48	
		%+	90.9	77.8	0.0	0.0	N/A	68.6	
HER2	Negative	N	22	2	0	7	N/A	31	N/A
		%+	100.0	5.6	0.0	100.0	N/A	44.3	
	Positive	N	0	34	5	0	N/A	39	
		%+	0.0	94.4	100.0	0.0	N/A	55.7	
Ki67	<%14	N	22	17	3	6	N/A	48	p=0.365
		%+	100.0	58.6	75.0	85.7	N/A	77.4	
	>%14	N	0	12	1	1	N/A	14	
		%+	0.0	41.4	25.0	14.3	N/A	22.6	

+Percentage within the group.

CD133 expression were observed (Figs. 1-3).

ER, PR, HER2 Expressions and Ki67 Status

Only in 1 patient in the LA group (4.5%) and 3 patients in the LB group (8.3%) were ER^-. 2 patients in the LA group (9.1%) and 8 patients in the LB group (22.2 %) were PR^-. 34 patients in the LB group (94.4 %) were $HER2^+$. LB subtype (n=12, 41.4%) has the highest Ki67 index positivity (Ki67>14%). However, there was no

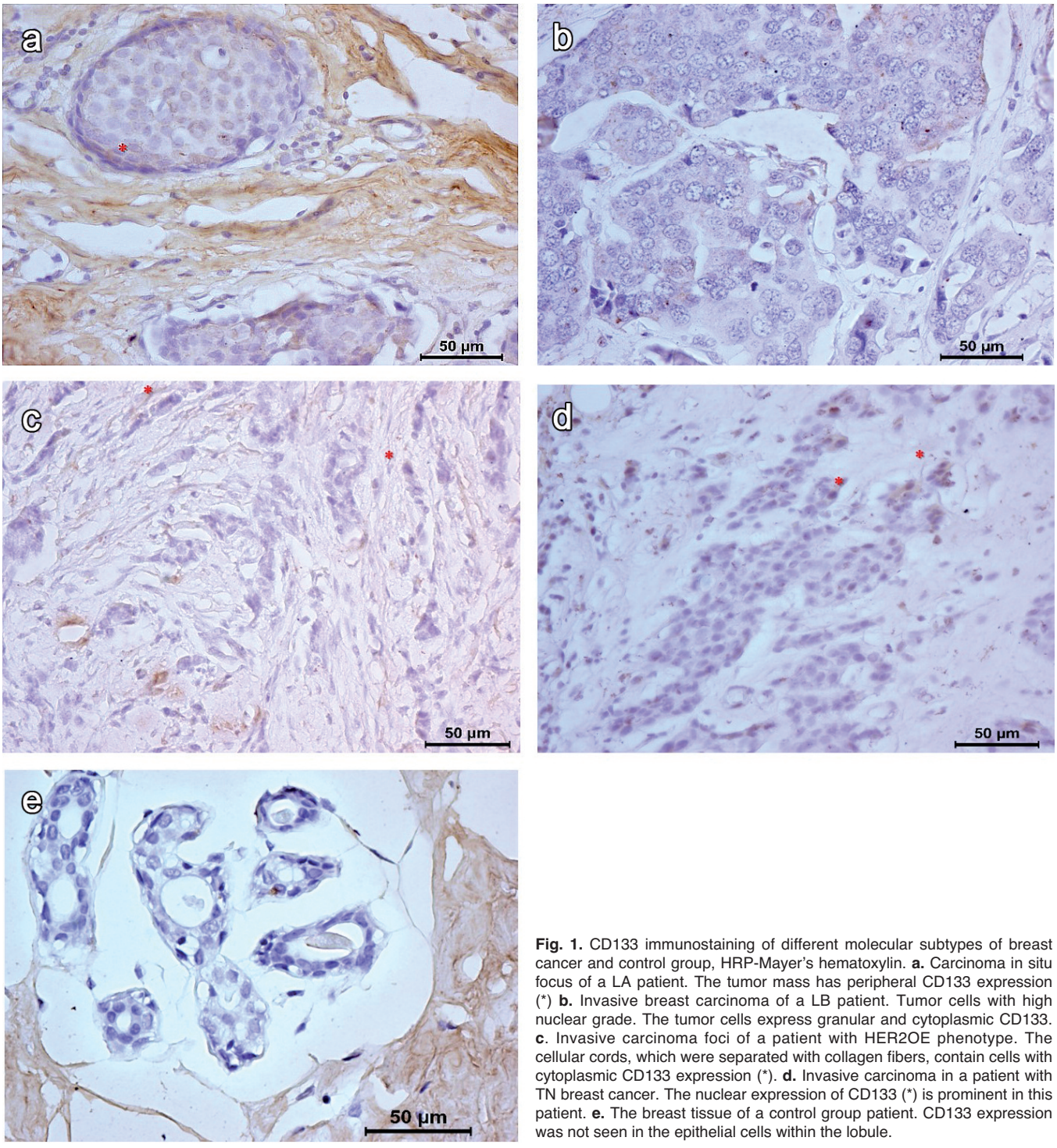

Fig. 1. CD133 immunostaining of different molecular subtypes of breast cancer and control group, HRP-Mayer's hematoxylin. **a.** Carcinoma in situ focus of a LA patient. The tumor mass has peripheral CD133 expression (*) **b.** Invasive breast carcinoma of a LB patient. Tumor cells with high nuclear grade. The tumor cells express granular and cytoplasmic CD133. **c**. Invasive carcinoma foci of a patient with HER2OE phenotype. The cellular cords, which were separated with collagen fibers, contain cells with cytoplasmic CD133 expression (*). **d.** Invasive carcinoma in a patient with TN breast cancer. The nuclear expression of CD133 (*) is prominent in this patient. **e.** The breast tissue of a control group patient. CD133 expression was not seen in the epithelial cells within the lobule.

statistically significant difference among the cancer groups regarding Ki67 index positivity (p=0.365) (Table 1).

The assessment of CD133, CXCR1 expression statuses and M27MP rations

There was a statistically significant difference among the groups in terms of CD133 H-Score (p=0.004). Significant differences were found between LA-control and LB-control group pairs (p=0.010 and p=0.002, respectively). While LB group had the highest median value (47.151, IQR=47.32425), the control group had the lowest (12.8290, IQR=10.7530). Although there were no statistically significant differences, the

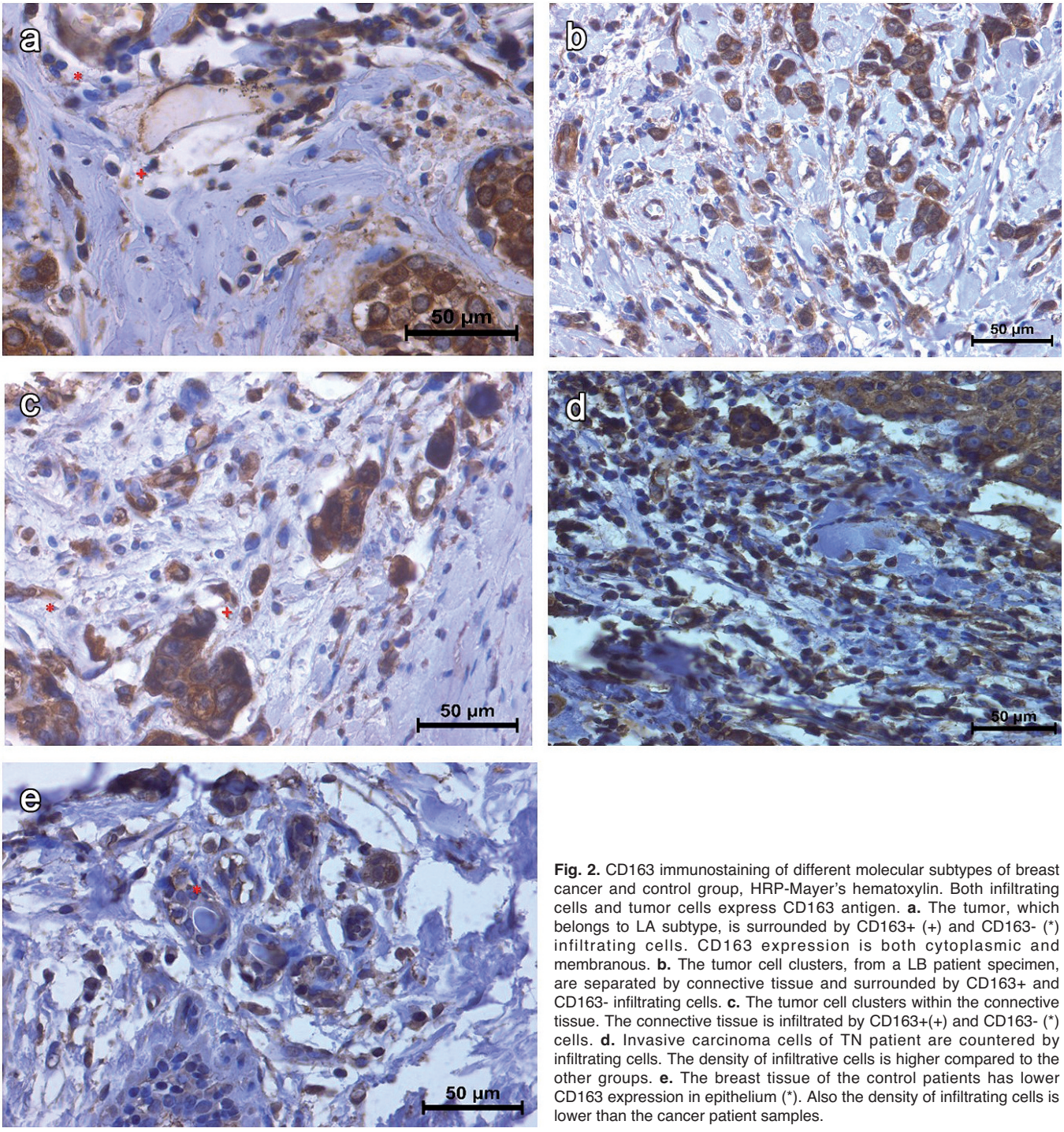

Fig. 2. CD163 immunostaining of different molecular subtypes of breast cancer and control group, HRP-Mayer's hematoxylin. Both infiltrating cells and tumor cells express CD163 antigen. **a.** The tumor, which belongs to LA subtype, is surrounded by CD163+ (+) and CD163- (*) infiltrating cells. CD163 expression is both cytoplasmic and membranous. **b.** The tumor cell clusters, from a LB patient specimen, are separated by connective tissue and surrounded by CD163+ and CD163- infiltrating cells. **c.** The tumor cell clusters within the connective tissue. The connective tissue is infiltrated by CD163+(+) and CD163- (*) cells. **d.** Invasive carcinoma cells of TN patient are countered by infiltrating cells. The density of infiltrative cells is higher compared to the other groups. **e.** The breast tissue of the control patients has lower CD163 expression in epithelium (*). Also the density of infiltrating cells is lower than the cancer patient samples.

median H-Score of TN group (26.2990, IQR=30.4970) was lower than LA (41.6670, IQR=23.5140), LB (47.151, IQR=47.32425) and HER2OE (44.35450, IQR=45.09625) groups. There was a statistically significant difference among three groups for CD133 expression percentages (p=0.006). The differences between LA-control (28.9310%, IQR=15.6825 and 11.980%, IQR=5.817, respectively) and LB-control groups (28.0825%, IQR=27.47550 and 11.980%, IQR=5.817, respectively) were found to be significant (p=0.008 and p=0.003). The highest median value occurred in HER2OE (28.8305%, IQR=30.8075) and

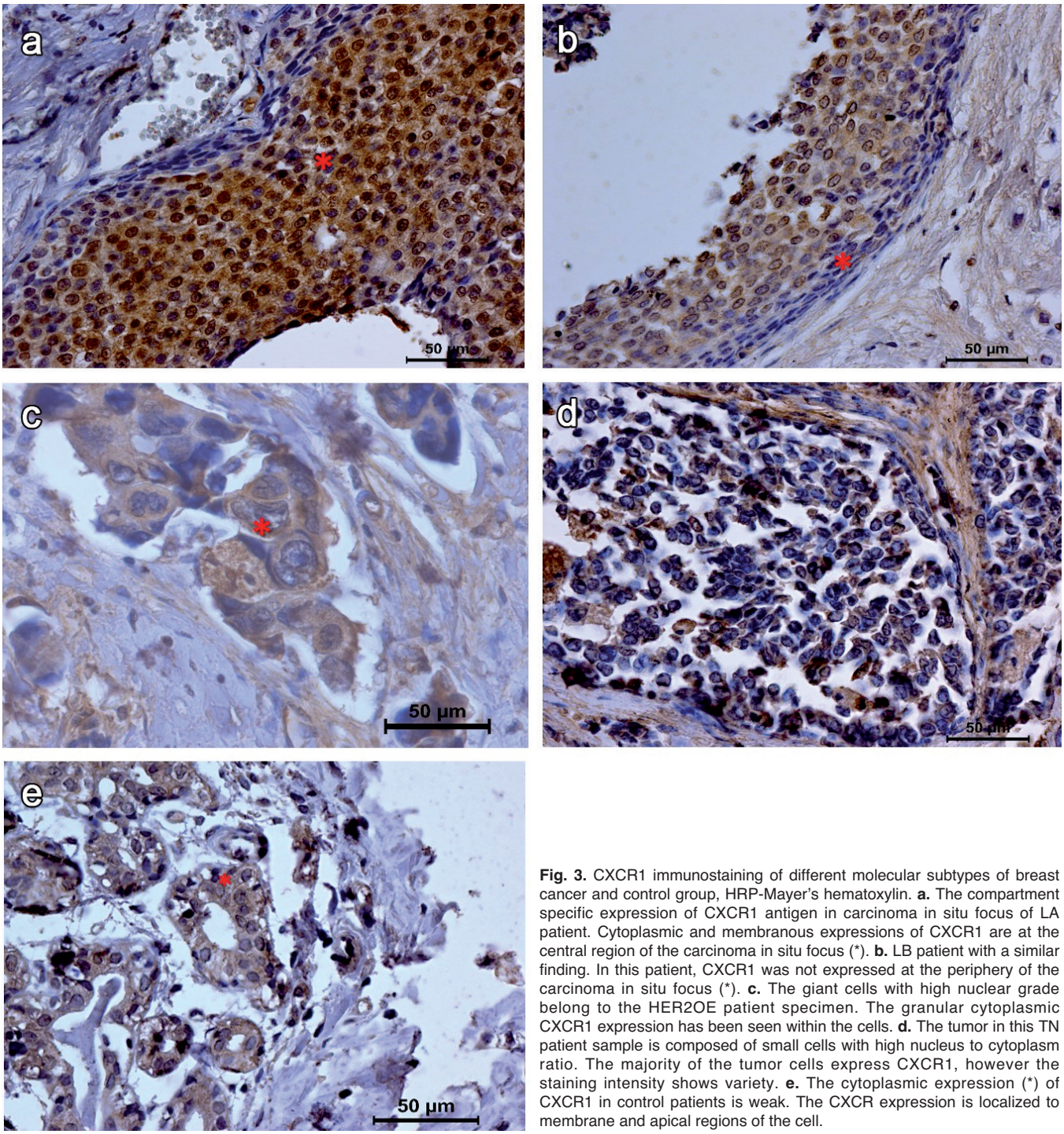

Fig. 3. CXCR1 immunostaining of different molecular subtypes of breast cancer and control group, HRP-Mayer's hematoxylin. **a.** The compartment specific expression of CXCR1 antigen in carcinoma in situ focus of LA patient. Cytoplasmic and membranous expressions of CXCR1 are at the central region of the carcinoma in situ focus (*). **b.** LB patient with a similar finding. In this patient, CXCR1 was not expressed at the periphery of the carcinoma in situ focus (*). **c.** The giant cells with high nuclear grade belong to the HER2OE patient specimen. The granular cytoplasmic CXCR1 expression has been seen within the cells. **d.** The tumor in this TN patient sample is composed of small cells with high nucleus to cytoplasm ratio. The majority of the tumor cells express CXCR1, however the staining intensity shows variety. **e.** The cytoplasmic expression (*) of CXCR1 in control patients is weak. The CXCR expression is localized to membrane and apical regions of the cell.

was higher than other cancer groups, although the difference was statistically insignificant. The lowest median value among the cancer groups was in TN (18.649%, IQR=20.352) (Table 2, Fig. 5).

There was a statistically significant difference among the groups regarding M2/MP ratio (p=0.022). The only significant difference in terms of M2/MP ratio was found between the LA and control groups (p=0.036). The highest M2/MP ratio was detected in control group (Median=70.00%, IQR=12.380), while the highest M2/MP ratio among cancer groups was found in HER2OE group (66.667%, IQR=12.921). The lowest median M2/MP ratio among all groups was found in LA (53.933%, IQR=12.851) (Table 2, Fig. 5).

In terms of CXCR1 H-Score, there was a statistically significant difference among the groups (p=0.002). Significant differences were found between the LB (median=145.5755, IQR=34.9817) -control (median= 86.5965, IQR=47.224) and TN (median=159.7200, IQR=41.412)-control group pairs (p=0.002 and p=0.002, respectively). TN had the highest CXCR1 H-Score among all groups. In addition, there was a statistically significant difference among the groups in terms of CXCR1 expression percentage (p=0.001). All cancer groups (LA, LB, HER2OE and TN) had significantly higher CXCR1 expression percentages compared to the control group (p=0.043, p=0.003, p=0.010 and p=0.003, respectively) (Table 2, Fig. 5).

The correlations between CD133, CXCR1 expression statuses and M2/MP ratios

There was a statistically significant but weak positive correlation (r=0.249, p=0.035) between CD133 and CXCR1 H-Scores. Also, a similar correlation was found between the CD133 and CXCR1 expression percentages (r=0.342, p=0.003). The correlations between the M2/MP ratio and CD133 H-score (r=-0.050, p=0.707), CD133 expression percentage (r=-0.081, p=0.536), CXCR1 H-score (r=0.010, p=0.941), CXCR1 expression percentage (r=0.028, p=0.830) were not significant (Table 3, Fig. 6).

The relationships between ER, PR, HER2, Ki67 statuses and CD133, CXCR1 expressions and M2/MP ratios

A statistically significant association has been found between ER expression positivity and CD133 H-Score values (p=0.015). The median H-score of ER^+ patients (median = 46.082, IQR=31.309) was higher than ER^- patients (median=26.299, IQR=26.382). A similar result was also found for CD133 expression between the ER^+ (median=30.097%, IQR=21.1925) and ER^- (median=18.6490%, IQR=19.973) patients (p=0.024). There was no significant relationship between ER status and M2/MP ratio (p=0.993), CXCR1 H-Score (p=0.274) and CXCR1 expression (p=0.233) (Table 4).

Moreover, a statistically significant relationship has been found between PR negativity and M2/MP ratio (p=0.007). The median M2/MP ratio of PR^+ patients (median = 57.020%, IQR=12.5585) was lower than PR^- patients (median= 65.094%, IQR=10.091). There was no significant association between the PR status and CD133 H-Score (p=0.794), CD133 expression (p=0.706), CXCR1 H-Score (p=0.367) and CXCR1 expression (p=0.234) values (Table 4).

Table 2. The statistical values for CD133 H-Score, expression percentage (%), M2/MP ratio (%), CXCR1 H-Score and expression percentage (%) for molecular subtypes of breast cancer.

				LA	LB	HER2OE	TN	Control	P-Value
CD133 H-Score	N		Valid	21	32	4	7	9	p=0.004*
		Median		41.66700	47.15100	44.35450	26.29900	12.82900	
	Percentiles		25	29.67850	27.45700	19.12475	22.93200	10.49350	
			75	53.19250	74.78125	64.22100	53.42900	21.24650	
CD133 Expression Percentage (%)	N		Valid	21	32	4	7	9	p=0.006*
		Median		28.93100	28.08250	28.83050	18.64900	11.98000	
	Percentiles		25	20.36600	18.48575	12.89575	17.64800	7.95350	
			75	36.04850	45.96125	43.70325	38.00000	13.77050	
M2/MP Ratio (%)	N		Valid	19	29	5	6	7	p=0.022*
		Median		53.93300	60.52600	66.66700	60.49200	70.00000	
	Percentiles		25	48.10100	55.03050	56.55350	50.75425	59.21100	
			75	60.95200	66.98750	69.47450	65.88400	71.59100	
CXCR1 H-Score	N		Valid	21	34	4	7	8	p=0.002*
		Median		135.5550	145.5755	141.5150	159.7200	86.5965	
	Percentiles		25	94.7640	128.7818	129.8413	140.0000	47.8688	
			75	161.1405	163.7635	177.0343	181.4120	95.0928	
CXCR1 Expression Percentage (%)	N		Valid	21	34	4	7	8	p=0.001*
		Median		83.4170	86.8405	92.5605	90.2770	54.6445	
	Percentiles		25	73.6090	78.9948	80.0075	79.0000	29.7023	
			75	89.6995	90.9268	96.9798	94.4830	60.6320	

*Statistically significant difference among the groups.

Furthermore, a significant relationship was detected between HER2 positivity and M2/MP ratio (p=0.016). The median M2/MP ratio of $HER2^+$ patients (61.92950%, IQR=10.6085) was higher than $HER2^-$ patients (56.00%, IQR=14.545). There were no significant relationships between the HER2 status and

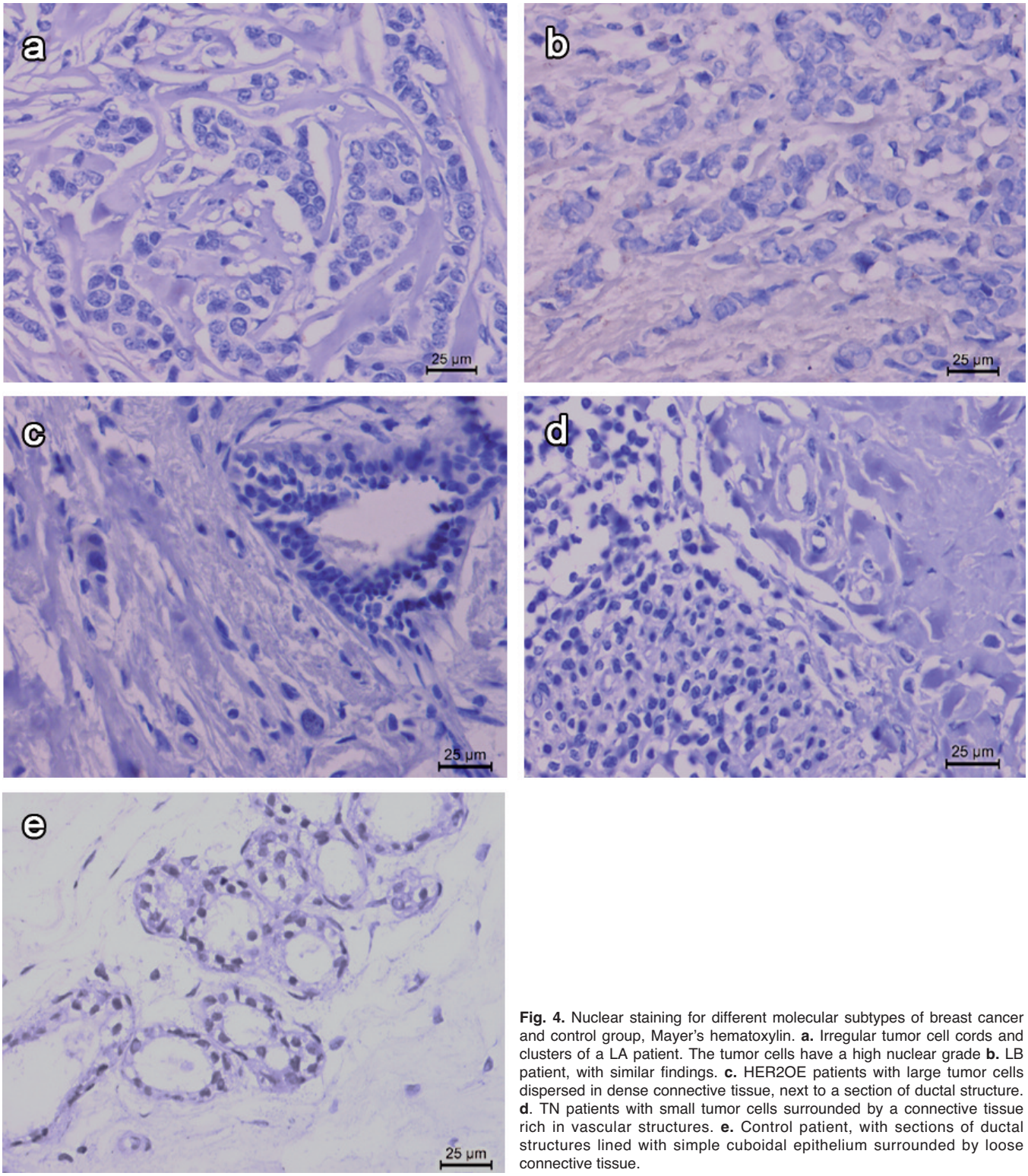

Fig. 4. Nuclear staining for different molecular subtypes of breast cancer and control group, Mayer's hematoxylin. **a.** Irregular tumor cell cords and clusters of a LA patient. The tumor cells have a high nuclear grade **b.** LB patient, with similar findings. **c.** HER2OE patients with large tumor cells dispersed in dense connective tissue, next to a section of ductal structure. **d**. TN patients with small tumor cells surrounded by a connective tissue rich in vascular structures. **e.** Control patient, with sections of ductal structures lined with simple cuboidal epithelium surrounded by loose connective tissue.

Table 3. The correlations between the CD133 H-Score, expression percentage, M2/MP ratio ,CXCR1 H-Score and expression percentage.

			CD133 H-Score	CD133 Expression Percentage (%)	M2/MP Ratio (%)	CXCR1 H-Score	CXCR1 Expression Percentage (%)
Spearman's rho	CD133 H-Score	Correlation Coefficient	1.000	0.984	-0.050	0.249	0.315
		Sig. (2-tailed)		0.000*	0.707	0.035*	0.007*
		N	73	73	60	72	72
	CD133 Expression Percentage (%)	Correlation Coefficient	0.984	1.000	-0.081	0.268	0.342
		Sig. (2-tailed)	0.000*		0.536	0.023*	0.003*
		N	73	73	60	72	72
	M2/MP Ratio (%)	Correlation Coefficient	-0.050	-0.081	1.000	0.010	0.028
		Sig. (2-tailed)	0.707	0.536		0.941	0.830
		N	60	60	66	60	60
	CXCR1 H-Score	Correlation Coefficient	0.249	0.268	0.010	1.000	0.843
		Sig. (2-tailed)	0.035*	0.023*	0.941		0.000*
		N	72	72	60	74	74
	CXCR1 Expression Percentage (%)	Correlation Coefficient	0.315	0.342	0.028	0.843	1.000
		Sig. (2-tailed)	0.007*	0.003*	0.830	0.000*	
		N	72	72	60	74	74

*Statistically significant correlation.

Table 4. The relationship between the CD133 H-Score, expression percentage, M2/MP ratio, CXCR1 H-Score, expression percentage and ER, PR, HER2 expression, Ki67 index statuses of the patients.

				CD133 H-Score	CD133 Expression Percentage (%)	M2/MP Ratio (%)	CXCR1 H-Score	CXCR1 Expression Percentage (%)
ER	NEGATIVE	N	Valid	15	15	15	15	15
		Median		26.29900	18.64900	60.00000	151.0960	89.7170
		Percentiles	25	21.95100	12.80500	50.63300	128.1550	76.8820
			75	48.33300	32.77800	66.66700	181.4120	94.4830
	POSITIVE	N	Valid	49	49	44	51	51
		Median		46.08200	30.09700	60.06750	144.5330	85.3930
		Percentiles	25	30.67800	21.49300	53.35475	120.5890	78.2410
			75	61.98700	42.68550	65.86600	161.7030	90.5670
p-Value				p=0.015*	p=0.024*	p=0.993	p=0.274	p=0.233
PR	NEGATIVE	N	Valid	20	20	19	21	21
		Median		43.28850	26.10150	65.09400	148.7410	88.8880
		Percentiles	25	24.81850	18.47800	58.16300	132.2635	79.2615
			75	66.13775	44.80925	68.25400	173.8880	93.2555
	POSITIVE	N	Valid	44	44	40	45	45
		Median		42.32450	28.36900	57.02000	143.9030	84.6160
		Percentiles	25	28.67250	18.94475	50.66700	117.9390	77.8935
			75	58.11475	40.76850	63.22550	162.1385	90.5960
p-Value				p=0.794	p=0.706	p=0.007*	p=0.367	p=0.234
HER2	NEGATIVE	N	Valid	30	30	27	30	30
		Median		40.03350	27.72600	56.00000	145.5480	84.0165
		Percentiles	25	26.23725	18.72475	48.27600	112.2780	75.6735
			75	54.07175	38.19100	62.82100	163.3405	90.3495
	POSITIVE	N	Valid	34	34	32	36	36
		Median		47.15100	28.08250	61.92950	147.3740	87.7280
		Percentiles	25	28.78300	19.76725	56.27000	132.9253	79.3355
			75	70.47550	45.22375	66.87850	166.1425	91.6408
p-Value				p=0.326	p=0.527	p=0.016*	p=0.347	p=0.385
Ki67	<%14	N	Valid	48	44	48	45	45
		Median		39.2805	27.412	53.9400	146.6180	846.160
		Percentiles	25	24.2628	18.6743	41.5963	117.9390	773.100
			75	52.1023	38.2835	62.8825	164.9780	908.025
	>%14	N	Valid	14	13	14	13	13
		Median		54.0075	39.597	60.0675	136.9900	86.9040
		Percentiles	25	26.6085	24.9035	52.5225	123.2715	76.2050
			75	74.5655	50.0825	66.5080	155.6575	91.0340
p-Value				p=0.152	p=0.065	p=0.116	p=0.595	p=0.874

*Statistically significant difference among the groups.

CD133 H-Score (p=0.326), CD133 expression (p=0.527), CXCR1 H-Score (p=0.347), CXCR1 expression (p=0.385) (Table 4).

Finally, Ki67 positivity was not related with CD133 H-Score (p=0.152), CD133 expression (p=0.065), M2/MP ratio (p=0.116), CXCR1 H-Score (p=0.595) and

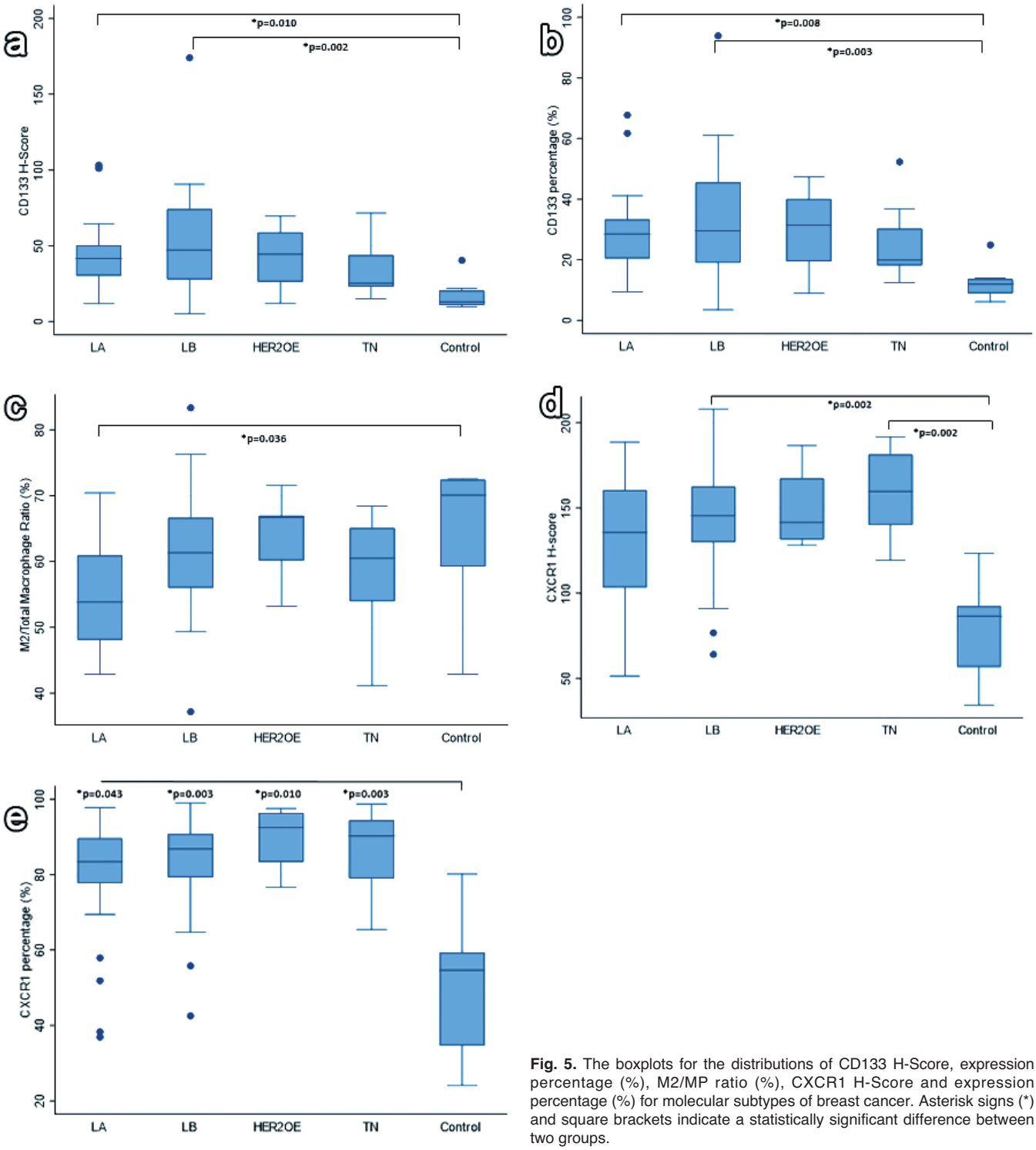

Fig. 5. The boxplots for the distributions of CD133 H-Score, expression percentage (%), M2/MP ratio (%), CXCR1 H-Score and expression percentage (%) for molecular subtypes of breast cancer. Asterisk signs (*) and square brackets indicate a statistically significant difference between two groups.

CXCR1 expression (p=0.874) (Table 4).

Chemotherapy statuses and their correlations with CD133, CXCR1 expressions and M2/MP ratios

Out of n=70 cancer patients, only n=6 (8.57%) patients received neoadjuvant chemotherapy prior to obtaining pathological specimens. Of the patients, n=38 (54.29%) did not receive chemotherapy prior to surgery. In n=26 (37.14%) patients, there was no record of chemotherapy history.

In terms of CD133 H-Score and CD133 expression percentage there was no statistically significant difference between the patients with and without neoadjuvant chemotherapy histories. (p=0.232 and p=0.265). Surprisingly, both CD133 H-score and expression percentage values were greater in patients without adjuvant chemotherapy history (median= 43.2225 and with IQR=19.595; median=29.4915 and with IQR=15.862, respectively versus median=33.333 and with IQR=21.84; median=24.074 and with IQR=13.503, respectively).

According to an analysis performed on the patients without neoadjuvant chemotherapy history, there was no significant difference among the groups in terms of CD133 H-Score and CD133 expression percentage (p=0.411 and p=0.447). While the highest CD133 H-score was detected in LB group (median=49.307 with IQR=30.825), the lowest values were in TN group (median=29.0895 with IQR=24.992). For the CD133 expression percentage, the highest value was in LA group (median=31.061 with IQR=10.302), and the lowest value was in TN group (median=21.096 with IQR=14.8615).

Discussion

In our study, patients with breast cancer were

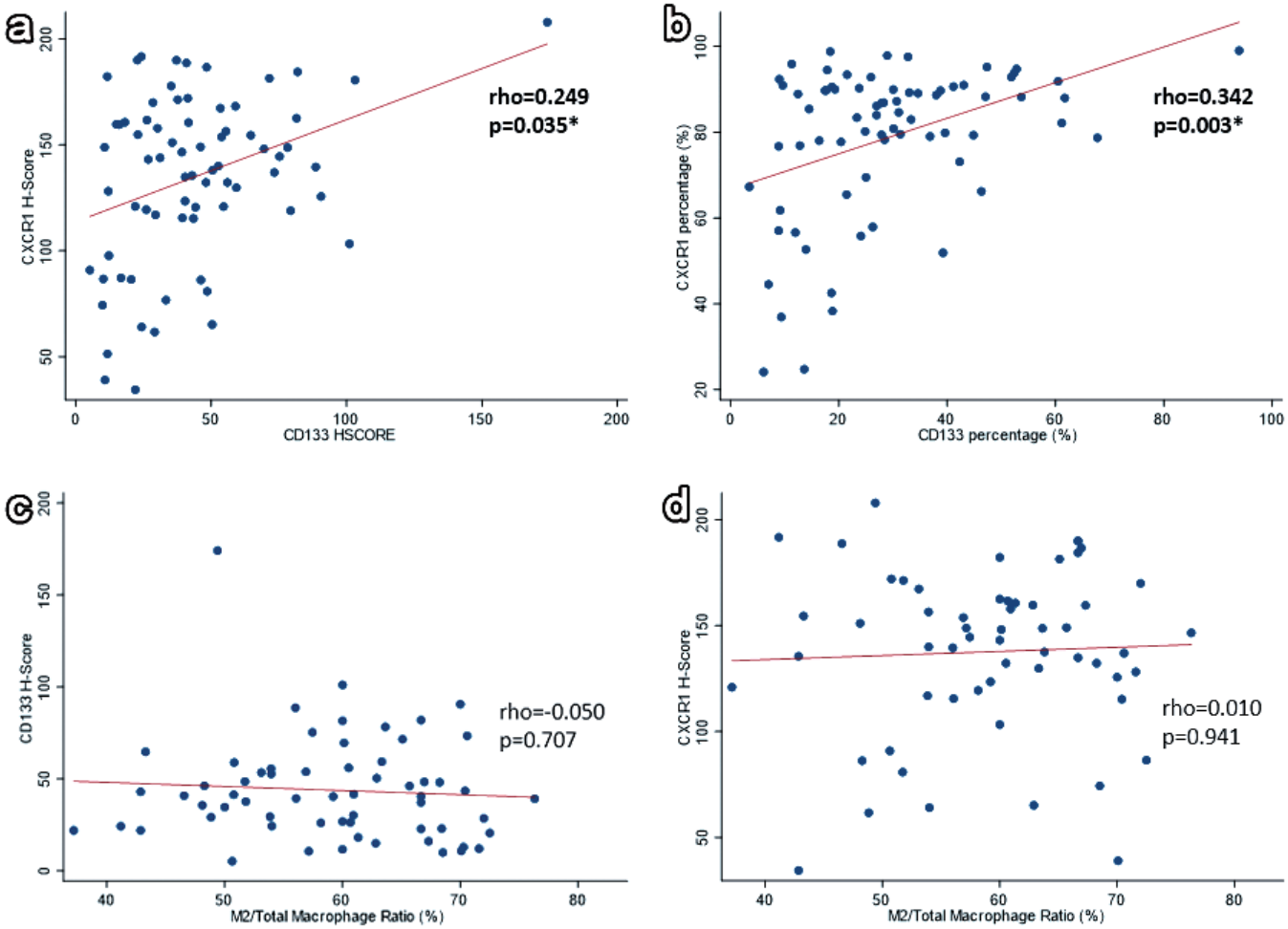

Fig. 6. The correlations between the CD133 H-Score, expression percentage, M2/MP ratio, CXCR1 H-Score and expression percentage. Asterisk sign (*) indicates a statistically significant correlation between two variables.

phenotypically categorized by their molecular subtypes. In our patient population, the majority of the patients were classified as LB subtype, which has poorer prognosis than LA subtype. Nevertheless, the greater part of the patients were classified in the LA subgroup according to a previous study (Howlader et al., 2014).

In our population, the oldest patients were in the LA group, which has a better prognosis and has lower risk for local recurrence. In contrast, the youngest patients belong to the LB subtype. This age difference among the groups can be explained by the late presentation of LA subtype (Voduc et al., 2010). The incidence of breast cancer in females is 122 times higher than in males (Ly et al., 2013). However, in our study, the ratio of the male patients to female patients was higher. In previous studies, the majority of the male breast cancer patients (44-75%) were classified as LA subtype (Kornegoor et al., 2012). Nevertheless, three male patients in our study were distributed equally to LA, LB and TN subgroups

In our study, histopathological features of molecular subtypes were nonspecific (Figs. 1-3). However, previous studies linked molecular subtypes to various histopathological features (Weigelt et al., 2010). Similar to an earlier study (Medrek et al., 2012), the density of the infiltrating cells in the TN subtype were higher in our specimens (Fig. 2d). The macrophages can form up to 50% of the tumor mass and similarly, the majority of infiltrating cells were macrophages in our sections. (Vinogradov et al., 2014). CD133 expression at the periphery of the tumor (Fig. 1a) is compatible with the location of stem cells within the ductal and alveolar basal compartment of normal breast tissue (Owens and Naylor, 2013). Since CXCR1 expression was detected in the central areas (Fig. 3a,b), the IL-8/CXCR1 pathway may be more active at the central region. Thus, IL-8 blockers like repertaxin (Ginestier et al., 2010) may have to reach the center of tumor for optimal efficiency. Cytoplasmic expression of CXCR1 in our study may be linked to tumor proliferation and increased vascularization of the surrounding tissue (Murphy et al., 2005). Nuclear CD133 expression (Fig. 1d) may be related to poor prognosis (Cantile et al., 2013). The high Ki67 positivity in LB group can be comparable with a previous study (Wang et al., 2016).

Our study showed that CD133 expression in cancer groups was higher than the control group in accordance with previous research (Tume et al., 2016). $CD133^{low}$ cells were characterized with high rate of proliferation and migration (Brugnoli et al., 2013). Moreover, circulating tumor cells (CTC) of breast cancer patients with luminal subtype showed high expression of CD133 prior to chemotherapy. However, the percentage of $CD133^{+}$ CTC increases after chemotherapy, and this increase is more prominent in non-luminal subtypes (Nadal et al., 2013). Another study showed high expression of CD133 in TN patients (Liu et al., 2013). Nevertheless, our study showed no difference in terms of CD133 H-score and expression percentage in patients with or without neoadjuvant chemotherapy. Similarly, no significant difference was found between the cancer groups composed of patients without neoadjuvant chemotherapy history. Thus, the use of CD133 as a specific marker for molecular subtypes remains controversial.

The high M2/MP ratio in control group can be explained with the high fat content of postmenopausal breast tissue. The myeloid derived suppressor cells in adipose tissue can induce the polarization of macrophages to M2 phenotype (Katz-Hanani et al., 2014). The dependency of the growth of TN cells on IL-6 and IL-8 (Hartman et al., 2013) can explain the high CXCR1 H-score in TN subtype. Furthermore, all cancer groups have higher CXCR1 expressions than the control group. Thus, all cancer groups may benefit from IL-8 blockers (Ginestier et al., 2010), but particularly TN patients may gain more from its beneficial effect.

Due to positive correlation between CD133 and CXCR1 expression in our study, CXCR1 may maintain the CD133 stem cell phenotype. A similar relation has also been previously found in pancreatic adenocarcinoma (Chen et al., 2014). Similar to our findings, correlations between the high M2 macrophage infiltration, PR negativity and HER2 positivity was shown previously (Kim et al., 2015) and can be explained by the poor prognosis of the non-luminal breast cancers (Chen et al., 2016).

The main limitation of this study was the use of immunohistochemistry on paraffin blocks, which are obtained for diagnostic purposes. In prospective studies, the immunohistochemistry results can be supported with qPCR and/or ELISA assays for measurement of antigen expression levels. In our study, we did not use the morphological classification system of breast cancer, since we wanted to focus on the distribution of the selected antigens among molecular subtypes of breast cancer. Another limitation for this study is the recruitment of breast cancer patients diagnosed in a range of 13 years, as a result of the retrospective nature of this study. A multi-centered prospective study design can narrow this time frame. Finally, the number of patients with a history of neoadjuvant chemotherapy in our patient population was limited. Prospective studies on the use of CD133 in different molecular subtypes of breast cancer by considering neoadjuvant chemotherapy statuses of patients, may be executed in larger cohorts.

In this study, we found a significant correlation between CD133 and CXCR1 expressions and compartment specific expressions of these antigens. Both CD133 and CXCR1 expressions in cancer groups were higher than the control group. On the other hand, the highest M2 macrophage ratio was found in the control group due to the high fat content of the postmenopausal breast tissue. In our study, the molecular subtypes showed heterogeneity in CD133, CXCR1 expression and $CD163^{+}$ TAM infiltration. We conclude, that LA, LB, HER2OE and TN molecular subtypes may have different subgroups, which exploit different signal pathways and possess distinct stem cell phenotypes.

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Can Ilgın MD, PhD presented this study on behalf of his colleagues Çomut MD, Sarıgül MD, Korkmaz PhD, Vardar MD and Müftüoğlu MD, PhD, in 'International Congress of Histochemistry and Cytochemistry-ICHC 2017' which has been held in Antalya, Turkey in 18-21st May 2017, as an oral presentation. The abstract and three figures (micrographs) of this study were published in the abstract book of the congress. ICHC 2017 organization allows the publication of this study as a scientific paper and does not state any conflict of interests including commercial interests or property rights.

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