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STROMAL CHANGES IN MOLECULAR SUBTYPES OF BREAST CANCER

311.02 - PATHOLOGICAL ANATOMY

Summary of PhD Thesis in Medical Sciences

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The thesis was elaborated at the Department of histology, cytology and embryology of the „Nicolae Testemitanu" State University of Medicine and Pharmacy in collaboration with the Department of Microscopic Morphology/Histology, the Angiogenesis Research Center of the „Victor Babes" University of Medicine and Pharmacy in Timisoara, Romania.

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The defence of the thesis will take place on 20.09.2023 at 14:00 within the „Nicolae Testemitanu" SUMPh, 165 Stefan cel Mare si Sfânt bld, office 205 at the meeting of the Commission for Public Defence of the doctoral thesis, approved by the decision of the Scientific Council of the Consortium *(minutes no.9 of 25.05.23).*

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CONTENT

CONCEPTUAL MARKINGS OF THE RESEARCH

Actuality and importance of the topic. Breast cancer is one of the most common malignant tumors in the world. According to GLOBOCAN data, in 2020 this tumor registered an incidence of 11.7% (2 206 771 new cases) of all cancers and a mortality of 6.9% (684 996 deaths) thus representing the first cause of death in women aged between 20 and 50 years. A higher incidence is seen in developed countries, a fact due to earlier diagnosis thanks to the implementation of mammography screening and the prevalence of hormonal and reproductive risk factors such as the early onset of menarche and the establishment of menopause at an older age, late first birth, small number of children, short breastfeeding, use of oral contraceptives, hormone replacement therapy, unhealthy lifestyle (alcohol and/or drug abuse, excessive body weight, lack of physical activity) [1, 2].

Breast cancer is a heterogeneous disease in terms of tumor morphological structure, therapeutic response, mode of distant metastasis and prognosis [3]. Standard analyzed parameters such as tumor size, histological grade, vascular invasion and lymph node metastases are useful but insufficient. Moreover, patients with apparently identical parameters can evolve completely differently, which makes the need for an individual approach imperative. The above-mentioned factors required the study of the molecular characteristics of the tumor and allowed the discovery of molecular subtypes by Perou et al. in 2000. These subtypes have different risk factors, progress differently and need to be addressed individually from a therapeutic point of view, and classification of tumors based on ER, PR and HER2 expression has become a routine procedure in the clinic [1, 4-5].

Over time, it has been noticed that even the molecular classification does not perfectly describe all entities, which is why it is necessary to identify new molecular markers [6]. Thus, the elements of the tumor stroma appeared on the scene, which until now were in the shadows because the study of cancer was mainly focused on the actual tumor cells. Although Paget proposed the „seed and soil" theory as early as 1889, suggesting that neoplastic cells („seeds") can form tumors only in the case of a favorable microenvironment („soil"), only recently have researchers begun to study the importance of the tumor stroma [7]. Changes produced in the stroma are now considered as one of the essential elements of tumorigenesis and tumor progression. Thus, it has been established that elements of the tumor microenvironment, such as immune cells, the extracellular matrix, can act synergistically by blocking antitumor immunity and stimulating cancer progression and metastasis. Thus, stromal elements have been shown to be prognostic factors, and some of these could be targeted therapeutically [8].

In this study we analyzed the expression of a series of cell markers in the tumor stroma depending on the molecular subtype of the neoplasm.

The aim of the work: Study of cell varieties and vascular elements in the stroma of breast carcinomas depending on the molecular subtype of the tumor in order to improve the morphological diagnosis and prognosis of breast cancer.

Objectives of the study:

1. Histological and immunohistochemical evaluation of cell varieties (CD68, S100, mast cell tryptase) in the stroma of breast carcinomas in different molecular subtypes.
2. Study of the particularities of blood (CD34) and lymphatic (D2-40) vascular networks in breast carcinomas according to the molecular subtype.

3. Analysis of the interrelationships between cellular elements, blood vessels and lymphatics *versus* the molecular subtype of the tumor.
4. Elucidation of the impact of various stromal elements on the diagnosis, prognosis and evolution of breast carcinoma.

Scientific research methodology. The specimens and patients' data were selected from the histopathological files in the archive of the Emergency Clinical Hospital, Pathological Anatomy Service, Arad, Romania. The histopathological profile of the tumors was done on hematoxylin and eosin-stained sections according to the standard procedure. Later, immunohistochemical staining was performed in order to establish the molecular profile of the tumor. For the study of stromal elements, microarray blocks were created from the remaining tissue material within each paraffin block. The entire immunohistochemical technique was performed automatically (Leica Bond-Max, Leica Biosystems, Newcastle Upon Tyne, UK), applying the following antibodies: anti-ER, anti-PR, anti-HER2, anti-CK5/6, anti-Ki-67, anti-CD68, anti-S100, anti-mast cell tryptase, anti-D2-40 and anti-CD34. The data were stored in an MS Excel 2010 database, and later analyzed using the SPSS program. Statistical tests included determination of mean, standard deviation, median, interquantile range, confidence interval, Spearman's correlation coefficient. The difference between two groups of variables was analyzed by applying the Mann-Whitney U test. We considered a $p \leq 0.05$ as statistically significant. The research was carried out in the Department of histology, cytology and embryology of the „Nicolae Testemitanu" State University of Medicine and Pharmacy and in the Department of Microscopic Morphology/Histology, Angiogenesis Research Center of the „Victor Babes" University of Medicine and Pharmacy in Timisoara, Romania. The study was approved by the Ethics Committee of the „Nicolae Testemitanu" State University of Medicine and Pharmacy (no. 33/ 37/ 12.02.2018).

The novelty and scientific originality of the obtained results. For the first time, a comparative study of the molecular profile of breast carcinomas *versus* cellular (macrophages, mast cells, dendritic cells) and vascular (blood and lymphatic) elements in the tumor stroma was performed. For the first time, the degree of expression of some cell markers (CD68, S100, tryptase, CD34, D2-40) in intra- and peritumoral areas was evaluated in relation to the expression of ER, PR and HER2 markers. Quantitative and qualitative changes in the cellular and vascular components of the tumor stroma in luminal and non-luminal subtypes have been described.

Applied scientific problem solved. In this paper, the possibility of the existence of the relationship between the differential expression of markers of some cellular elements in the tumor stroma and the evolution of luminal *versus* non-luminal breast carcinomas was analyzed.

Theoretical significance. Quantitative and qualitative assessment of stromal elements in breast carcinomas allows to complete the existing knowledge about the role of stroma in the evolution and progression of breast neoplasias, with emphasis on the differences between the luminal and non-luminal group.

The applicative value of the work. The work confirms the usefulness of applying histological methods and immunohistochemical staining in the diagnosis and evaluation of stroma changes in breast neoplasia.

Main scientific results submitted for defending:

1. The tumor microenvironment represents one of essential elements in the structure of breast carcinomas, influencing their growth and progression. The interrelationships between epithelial cells and stromal structures are complex, stimulatory or inhibitory, thus management of the stroma is imperative to potentiate or slow the expansion of neoplastic cells.

2. In luminal breast carcinomas, the density of mast cells in the peritumoral areas prevails over that of macrophages and dendritic cells showing a statistically significant positive correlation. In ER+ and PR+ tumors, the intratumoral areas showed a higher density of mast cells compared to the peritumoral ones, thus hormone-positive tumors show chemoattractiveness for tryptase+ cells, stimulating their migration.
3. Most hormone-independent breast carcinomas showed a high density of macrophages in both intratumoral and peritumoral stroma. In ER+ and PR+ carcinomas the density of CD68+ cells registered low values.
4. Loss of estrogens/progesterone receptors is accompanied by increased density of peritumoral S100+ cells. S100 positivity of cancer cells suggests their antigenic modulation, with property transfer and „mimicry" or may be the consequence of the fusion of dendritic cell with the neoplastic cell.
5. Mast cells are the cellular elements capable of modulating the stroma of breast carcinomas, being involved in angiogenesis and lymphangiogenesis, the high density of mast cells being accompanied by the decrease in the number of dendritic cells and macrophages.
6. In poorly differentiated breast carcinomas, the density of blood and lymphatic vessels is higher, with predilection in the peritumoral areas, thus creating favorable conditions for tumor expansion. Breast carcinomas expressing receptors for estrogens, progesterone or the HER2 protein do not show statistically significant correlation with vascular density.

Implementation of scientific results: The obtained results were implemented in the didactic and scientific activity of the Department of histology, cytology and embryology and the Morphology Laboratory of SUMPh „Nicolae Testemitanu".

Approval of results: The results obtained and the basic concepts of the thesis were presented and discussed in the following scientific events: Days of the State University of Medicine and Pharmacy „Nicolae Testemitanu" from the Republic of Moldova (Chisinau, 2019); The Congress devoted to the 75th anniversary of the founding of the „Nicolae Testemitanu" State University of Medicine and Pharmacy from the Republic of Moldova (Chisinau, 2020); The international scientific conference dedicated to the 75th anniversary of the founding of the „Nicolae Testemitanu" State University of Medicine and Pharmacy from the Republic of Moldova (Chisinau, 2020); International medical congress for students and young doctors MedEspera (Chisinau, 2020); Biomedical and Health Research Annual Scientific Conference: Quality, Excellence and Performance. Days of the State University of Medicine and Pharmacy „Nicolae Testemitanu" from the Republic of Moldova (Chisinau, 2021).

The results of this study were discussed and approved at the meeting of the Department of histology, cytology and embryology of SUMPh „Nicolae Testemitanu" (minutes no. 8 of 11.04.23) and the Scientific Profile Seminar 311. Anatomy and morphology; 351. Interdisciplinary medicine (minutes no. 2 of 23.05.23).

Publications on the topic of the thesis. 9 scientific papers were published on the topic of the thesis, among which: 1 article in international journals indexed in SCOPUS / PubMed; 5 articles in journals from the National Register of profile journals; 1 article in the materials of international scientific conferences; materials at national and international conferences – 2. At the same time, 1 innovator certificate and 1 act of practical implementation of scientific results were obtained.

Summary of the chapters of the thesis. The work includes list of abbreviations, introduction, 4 chapters, general conclusions and practical recommendations. The bibliography includes 209

sources, followed by 7 annexes, the statement regarding the assumption of responsibility, the author's CV. In the introduction, the actuality and importance of the problem addressed in the thesis, the purpose, the objectives, the general research methodology, the scientific novelty, the theoretical importance and the applied value of the work, the approval of the study results were described. Chapter 1 includes the literature review, chapter 2 – research material and methods, chapter 3 – obtained data, chapter 4 – synthesis and analysis of the obtained results.

Key words: ER, PR, HER2, molecular subtypes, microenvironment, intratumoral stroma, peritumoral stroma

THESIS CONTENT

1. TUMOR STROMA IN DIFFERENT MOLECULAR VARIANTS OF BREAST CARCINOMA

In this chapter, the etiological factors in the development of mammary carcinomas, the histology and the molecular profile of the mammary gland in both normal and malignant transformation were described. The role of stromal elements (cellular and vascular) in triggering and sustaining the neoplastic process, the controversies related to them, the possibility of therapeutic targeting was also analyzed.

2. MATERIALS AND RESEARCH METHODS

2.1. Study design, characteristics of the research group

The given study was a retrospective one based on the consultation of the histopathological bulletins and the microscopic evaluation of 111 cases of mammary gland carcinomas. The cases included in the study were selected during 2018-2019, within the Emergency Clinical Hospital, Pathological Anatomy Service, Arad, Romania. The biopsy fragments were obtained after partial or total mastectomy from patients aged between 37 and 85 years, the mean age being 67.9±8.6 years, and the median 64 years (25-75% IQR 59-73).

The patients did not undergo radio- or chemotherapy until the operation. All procedures were performed according to the principles of the Declaration of Helsinki. The research was carried out in the Department of histology, cytology and embryology of the „Nicolae Testemitanu" State University of Medicine and Pharmacy and in the Department of Microscopic Morphology/Histology, Angiogenesis Research Center of the „Victor Babes" University of Medicine and Pharmacy in Timisoara, Romania.

2.2. Histological method

The histopathological profile was performed on hematoxylin and eosin stained sections according to the standard procedure. Specimens were fixed in 10% buffered formalin solution for 48 hours and traditionally embedded in paraffin after dehydration and clarification procedures.

Subsequently, 4 μm thick sections were cut from each block for hematoxylin and eosin staining. Leica RM2245 microtome (Leica Biosystems, Newcastle Upon Tyne, UK) was used for sectioning. Sections were mounted on Surgipath X-tra Adhesive silanated slides (Leica Biosystems, Newcastle Upon Tyne, UK) and dried at 37°C for 20–30 minutes. These slides were used to determine the histological type of the tumor and to evaluate the degree of differentiation (Notthingam

score). Cases were examined by 3 independent morphopathologists, and suitable sections were chosen for immunohistochemical staining.

2.3. Immunohistochemical method

Next, sections were cut from each block for molecular subtype determination. Then, the remaining tissue material within each paraffin block was used to create microarray tissue blocks using TMA Grand Master (3DHISTECH Ltd., Budapest, Hungary). 1-4 tissue fragments from the most representative regions of the tumor were extracted from each block. Thus, 11 TMA blocks were obtained, in total containing 211 tissue fragments.

A series of 4 μm sections were cut from each microarray block. Leica RM2245 microtome (Leica Biosystems, Newcastle Upon Tyne, UK) was used for sectioning. Sections were mounted on Surgipath X-tra Adhesive silanated slides (Leica Biosystems, Newcastle Upon Tyne, UK). The immunohistochemical technique was performed using the automatic Leica Bond-Max (Leica Biosystems, Newcastle Upon Tyne, UK). Unmasking was performed using PT-link, at 97°C for 20 min and Bond Epitope Retrieval Solution 1 and 2, solutions with pH 6 and pH 9 respectively (Leica Biosystems, Newcastle Upon Tyne, UK). To block endogenous peroxidase, 3% hydrogen peroxide was used for 5 minutes. This step was followed by incubation with the primary antibody: ER, PR, HER2, CK5, EGFR, Ki-67 – to determine the molecular subtype; CD68 for the study of macrophages; S100 to highlight DC; mast cell tryptase for MCs study; D2-40 for determining lymphatic vessels; CD34 for the study of blood vessels.

The chromogen used was 3,3-diaminobenzidine tetrachloride for 10 minutes. Nuclei were counterstained with Mayer's hematoxylin, 5 minutes. Mounting was performed automatically with Leica CV5030 (Leica Biosystems, Newcastle Upon Tyne, UK) using ENTELLAN type permanent mounting medium namely Leica CV Mount (Leica Biosystems, Newcastle Upon Tyne, UK). Due to some considerations (insufficient tissue material, detachment of sections from the slides, etc.), immunohistochemical staining was successfully performed on less tissue fragments. Subsequently, only the cases in which the molecular profile was determined by IHC were selected. Details of the immunohistochemical technique can be found in table 1.

Table 1. **Antibodies, dilutions, detection system, unmasking, incubation**

Antibodies	Source	Clone	Dilution	Detection system	Unmasking	Incubation time
ER	Leica Bond (Leica Biosystems, Newcastle UponTyne, UK)	6F11	ready-to-use	Bond Polymer Refine Detection System	20 min ER 2	30 min
PR		16				
CK5		XM26				
CD 34		QBEnd10				
Mast cell tryptase		10D11				
CD68		514H12				
S100		polyclonal			20 min ER 1	
Ki-67		MM1			10 min ER 1	
HER2	Novocastra (Leica Biosystems, Newcastle UponTyne, UK)	CB11			20 min ER 2	
EGFR		EGFR.113				
D2-40	Abcam (Cambridge, UK)	gp36	1:40		10 min ER 1	1 h

Note*: *ER 1 = Bond Epitope Retrieval Solution 1, pH6; ER 2 = Bond Epitope Retrieval Solution 2, pH9.

2.4. Quantification methods

Assessment of molecular subtype

To classify the breast carcinoma specimens according to the molecular profile, the expression of ER and PR hormone receptors, Ki-67, HER2, CK5 and EGFR proteins were analyzed. Positivity for each immunohistochemical marker was determined microscopically by 3 morphopathologists who reviewed each slide and assigned a score. Ki-67 was rated semi-automatically.

Hormone receptors (**ER and PR**) were evaluated according to the Allred score. **HER2** protein was assessed according to the recommendations of the American Society of Clinical Oncology. For the evaluation of the **Ki-67** marker, the semi-automatic method proposed by Suciu et al was applied. [6]. Thus, immunolabeled and negative nuclei from tumor cells were counted and the percentage of stained ones was calculated. The positivity threshold was 14%. Based on the 2017 St Gallen International Expert Consensus, the immunohistochemical profile of the molecular subtypes was as follows:

ER+ and/or PR+, HER2-, Ki-67 < 14% for luminal A subtype;

ER+ and/or PR+, HER2+ and/or Ki-67 > 14% for luminal B;

ER-, PR-, HER2+ for HER2+ subtype.

Specimens lacking staining for ER, PR, and HER2 were classified as triple-negative subtype.

Identification and quantification of stromal elements

All analyzed stromal elements were quantified by the hot-spot method: areas with the highest density of targeted elements were first detected by scanning the sections at 10× ob. using the Axio Imager A2 microscope (Carl Zeiss, Germany). Later, 2-3 consecutive fields from the tumor areas and the peritumoral stroma were analyzed, calculating the arithmetic mean.

Macrophages were identified due to the cytoplasmic expression of the CD68 marker. We quantified the macrophages in 2 consecutive fields from the intratumoral and the peritumoral stroma at 20× ob., later calculating the arithmetic mean. We applied the following score:

"0" – no CD68+ cells were observed in the field;

"+1" – up to 25% of CD68+ cells in the field;

"+2" – 25-50% CD68+ cells in the field;

“+3” – more than 50% CD68+ cells in the field of view.

Mast cells and dendritic cells were identified due to the expression of specific markers: tryptase and S100 protein, respectively. We analyzed their distribution in the tumor island and peritumoral beaches by directly counting the cells in the respective areas. 3 microscopic fields were identified by the hot-spot method for each zone and analyzed at 40× ob. Afterwards, the arithmetic mean of the 3 values was calculated.

Blood vessels were detected due to the membraneous expression of CD34, a marker of endothelial cells and hematopoietic cell progenitors. Thus, initially we located the most vascularized areas of the tumor (CD34it) and around the tumor (CD34pt). We analyzed 3 fields from the intratumoral and peritumoral regions with the 20× ob. and counted CD34-positive blood vessels, with MVD representing the arithmetic mean of the 3 values.

Lymphatic vessels were detected due to the expression of D2-40 by the lymphatic endothelium. They were counted in 3 consecutive fields from the intra- and peritumoral areas, at 20× ob., subsequently calculating the arithmetic mean. This value represented the LVD.

2.5. Statistical analysis

The data were stored in an MS Excel 2010 database, and later imported and analyzed using the SPSS program (SPSS Statistics 23.0; IBM, Chicago, IL, USA). Mean, standard deviation, median, IQR, CI were determined. We applied the Spearman correlation test (r_s), and the values were considered statistically significant in case of a $p \leq 0.05$. The degree of association between the variables was deciphered, based on the latest recommendations in the field, according to Lovakov and Agadullina (2021) as follows: $r_s < 0.12$ - very weak correlation, $0.12 \leq r_s < 0.24$ - weak correlation, $0.24 \leq r_s < 0.41$ - moderate correlation, $r_s \geq 0.41$ - strong correlation.

Variables were grouped depending on the purpose pursued (eg. luminal/non-luminal subtype, low/high density, etc.). The numerical values of the stromal elements, based on studies in the field, were grouped using the median as the threshold (cut-off). Later, the difference between two groups of variables was analyzed by applying the Mann-Whitney U test. The cases where the molecular profile was known were statistically analyzed.

3. QUANTITATIVE AND QUALITATIVE CHANGES OF STROMAL ELEMENTS IN DIFFERENT MOLECULAR SUBTYPES OF BREAST CARCINOMA

3.1. The histopathological profile of the tumors included in the study

In the study performed, most tumors were moderately differentiated (G2) (78 cases). Only one case of highly differentiated tumor (G1) was detected, and 27 were poorly differentiated (G3). 71.7% of tumors (76 cases) were classified as invasive ductal carcinomas, NST type. The majority of G3 tumors (23 out of 27/ 85.2%) were invasive ductal carcinomas, NST type. The distribution of cases according to the histological type and tumor's grade is shown in table 2.

Table 2. **Histopathological classification of the tumors included in the study**

		Tumor grade			Total	
Histological type		**G1**	**G2**	**G3**	**N (%)**	**95% CI**
Invasive ductal carcinoma, type NST		1	52	23	**76 (71.69%)**	0-88.77
Invasive lobular carcinoma		-	4	-	**4 (3.77%)**	0-7.07
Mucinous carcinoma		-	2	-	**2 (1.88%)**	0-3.53
Medullary carcinoma		-	8	-	**8 (7.54%)**	0-14.14
Intraductal papillary carcinoma		-	1	-	**1 (0.94%)**	0-1.76
Carcinoma *in situ*:	lobular	-	2	-	**2 (1.88%)**	0-3.53
	ductal	-	-	1	**1 (0.94%)**	0-1.76
Mixed carcinoma:						
Invasive ductal + invasive lobular		-	5	3	**8 (7.54%)**	0-8.91
Invasive ductal + medullary		-	1	-	**1 (0.94%)**	0-1.76
Invasive ductal +mucinous		-	3	-	**3 (2.83%)**	0-5.30
Total		**1 (0.94%)**	**78 (73.58%)**	**27 (25.47%)**	**106**	

Note: *the table shows only the cases in which the molecular subtype was determined.*

3.2. Molecular profile of the tumors included in the study

The most frequent molecular subtype determined by immunohistochemical staining was luminal B/ HER2+. Most tumors of this molecular subtype were invasive ductal carcinomas (36

cases), 7 cases were medullary carcinomas, 1 case – papillary, 1 case – DCIS, the others – of mixed type.

11 out of 12 TNBC tumors were invasive ductal carcinomas, with 2/3 of cases (8 out of 12) being poorly differentiated. Among the HER2+ cases, only 1 tumor was medullary carcinoma, the rest were ductal invasive carcinomas, type NST. 8 out of 16 cases of HER2+ tumors were poorly differentiated. Luminal A type tumors included the only case of well-differentiated G1 tumor, and 14 out of 19 cases were moderately differentiated. Infiltrative lobular carcinomas were either luminal A type (1 case) or luminal B/ HER2+ type (3 cases).

3.3. CD68-positive macrophages in breast carcinomas

Macrophages, colored brown, presented varied aspects from a morphological point of view. In some specimens they appeared as small, rounded cells with few or no cytoplasmic extensions. The cytoplasm was uniformly, moderately or intensely stained. At the same time, amoeboid-looking macrophages were highlighted, with numerous cytoplasmic processes on the cell surface. Another group of macrophages was characterized by a strong cytoplasmic granulation. In addition to those mentioned, spindle-shaped macrophages were also present, some multinucleated, which were characterized by an elongated cell body with cytoplasmic extensions at the opposite poles.

Topographically, both intratumoral and peritumoral CD68-positive macrophages were determined in all sections. The latter were concentrated around tumor cells, perivascular and/or in areas of invasion (figure 1a, b).

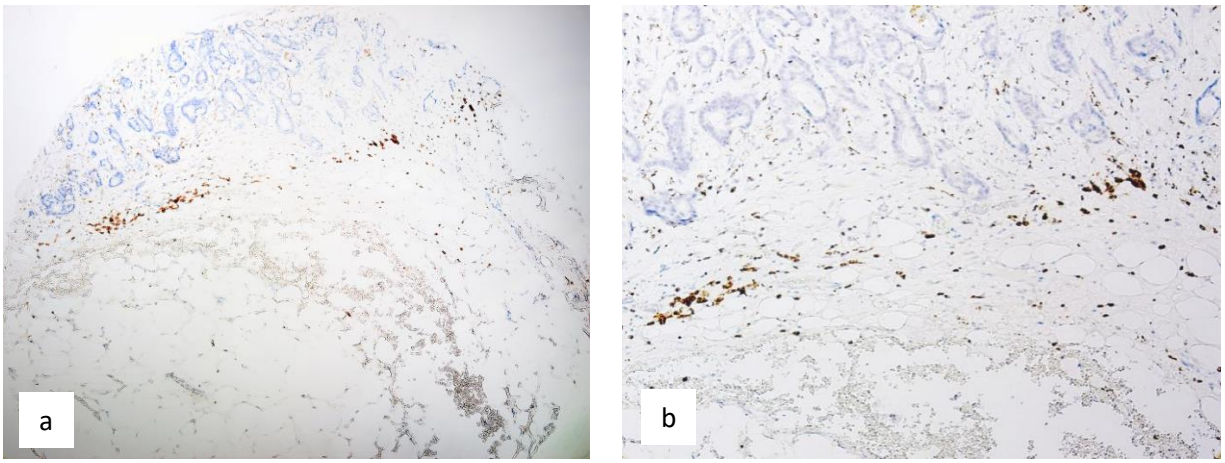

Figure 1. **Invasive ductal carcinoma, type NST. Macrophages at the border of the invasion area (a, b) (a - 10× ob., b - 20× ob.). Immunoreaction for CD68, DAB.**

In most tumors, both CD68it and CD68pt showed low density, with +1 score in 38 (48.1%) and score <1 in 43 cases (54.5%). The +2 score was frequently given to luminal tumors. Peri- and intratumoral CD68+ macrophages were almost equally distributed in luminal B/ HER2+ and TNBC tumors. The highest density of CD68it macrophages was recorded in TNBC tumors, and of CD68pt – in HER2+ tumors. It should be noted that 10 out of the 14 tumors that scored +3 for CD68pt expressed the HER2 protein. Also, 5 out of 8 tumors scored +3 for CD68it expressed HER2 protein. The largest gap between intra- and peritumoral macrophages occurred in HER2+ tumors. The distribution of intra- and peritumoral macrophages showed no differences in G2 tumors; intratumoral macrophages slightly prevailed over peritumoral ones in G3 tumors (figure 2).

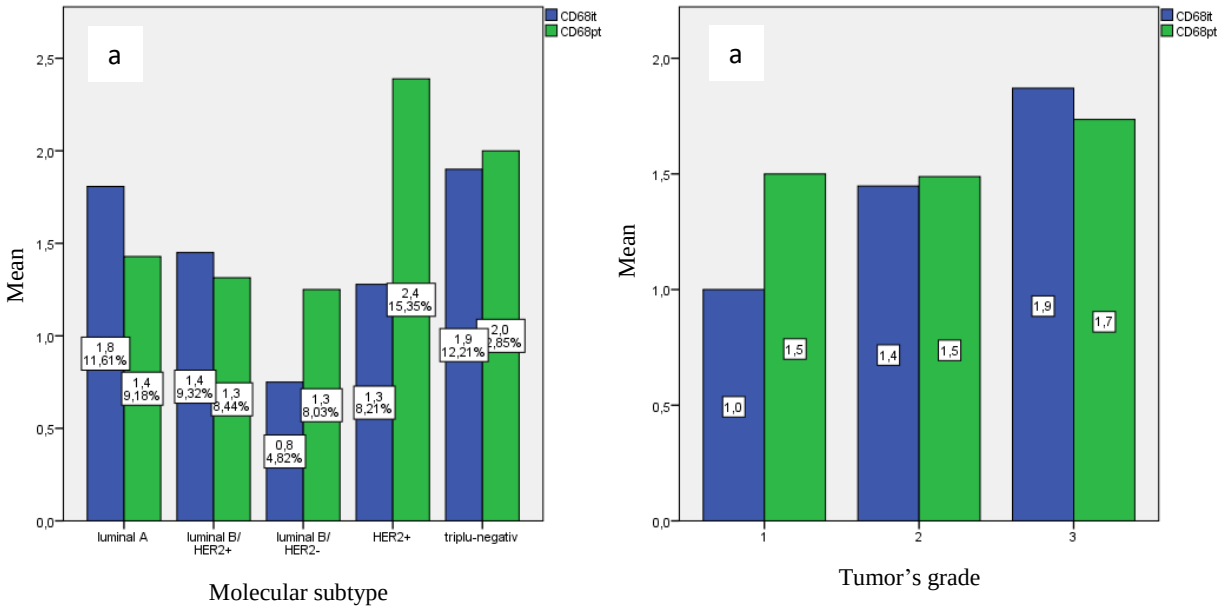

Figure 2. **Density of CD68+ macrophages depending on (a) molecular subtype and (b) tumor grade.**

Statistical analysis showed statistically significant inverse correlations between CD68pt and ER (r_S= -0.316, p= 0.005) and PR (r_S= -0.280, p= 0.012) hormone receptors. Also, CD68pt correlated with the molecular subtype of the tumor (r_S= 0.267, p= 0.017). CD68it were influenced by tumor's grade (r_S= 0.233, p= 0.039).

Subsequently, the cases were grouped into 2 categories: luminal and non-luminal. To compare CD68pt and CD68it in luminal *versus* non-luminal subtypes, we performed the Mann-Whitney U test (table 3). The obtained data confirmed the influence of the expression of hormonal receptors on the dynamics of CD68pt (U= 265, p= 0.0002).

Table 3. **CD68pt and CD68it in luminal *versus* non-luminal breast carcinomas**

	CD68it	**CD68pt**
Mann-Whitney U	562,000	265,000
Wilcoxon W	2392,000	2095,000
Z	-,096	-3,699
p	,924	**,0002**

Note: *the luminal group comprised the luminal A, B/ HER2+ and B/ HER2- subtypes; the non-luminal group included HER2+ and triple-negative tumors; CD68it – intratumoral CD68+ cells, CD68pt – peritumoral CD68+ cells.*

3.4. Tryptase-positive mast cells in mammary carcinomas

Microscopically, mast cells have been characterized as large cells of an ovoid shape, round nucleus, often invisible due to the high content of granules with mediators in the cytoplasm. Such mast cells had a dark, intensely coloured cytoplasm and were classified as granular (figure 3a). In contrast, in degranulated mast cells, the nucleus was visible, the cytoplasm became less stained, and granules could be seen in the ECM (figure 3b).

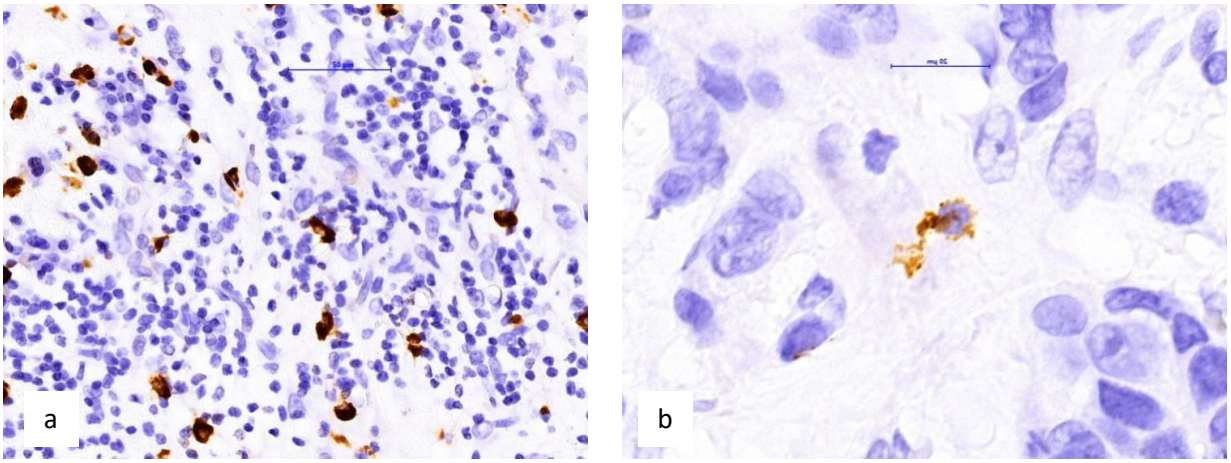

Figure 3. **(a) Invasive ductal carcinoma, type NST and (b) infiltrative lobular carcinoma. Mast cells: (a) granulated and (b) degranulated (a - 40× ob., b - 100× ob.). Immunoreaction for tryptase, DAB.**

Mast cells in the peritumoral stroma tended to „border" the islands of tumor cells, being located at the interface between the tumor area and the peritumoral conjunctive stroma. We could frequently observe peritumoral mast cells around the blood vessels or within the inflammatory infiltrate.

For a better appreciation of MCs infiltration, the data were subsequently dichotomized. Thus, the cases were divided into 2 groups: with a high density of MCs and with a low density, the median serving as a threshold (cut-off). For MCit, the mean and standard error was 3.54±0.61, with a median of 1.3 (25–75% IQR 0.3–3.9). In the case of MCpt – the mean and standard error were 12.26±1.39, and the median – 9.0 (25-75% IQR 3.3-15.9). 41 cases (51.2%) had a low intratumoral MC density (≤1.3). Likewise, 41 tumors (51.2%) had a low peritumoral MC density (≤9.0) (table 4). Among all cases, 29 tumors (36.2%) had a low density of both MCit and MCpt. 17 of them belonged to the luminal group. A high density of both MCit and MCpt was recorded in 27 tumors (33.8%), most of which were of the luminal type (23 tumors).

The study of cases according to molecular subtype showed that the highest densities, for both MCit and MCpt, were recorded within the luminal B/ HER2+ subtype, these tumors being moderately differentiated. The lowest densities were recorded in G3 tumors, HER2+ subtypes and TNBC, i.e. non-luminal. In all molecular subtypes, MCpt were more numerous than MCit.

Analyzing each molecular subtype separately, several statistically significant correlations were found between intra- and peritumoral MCs populations, as well as with ER and PR hormone receptors. Thus, in luminal A subtype, intratumoral MCs were influenced by PR expression (r_s= -0.703, p= 0.005). In luminal B molecular subtype, intratumoral MCs were dependent on ER (r_s= 0.350, p= 0.017), while peritumoral ones correlated with both hormone receptors (r_s= 0.322, p= 0.029 for ER and r_s= 0.308, p= 0.037 for PR). In this molecular subtype, as well as in the triple-negative one, the densities of the two MCs categories were interdependent (r_s= 0.590, p= 0.00001 and r_s= 0.687, p= 0.002, respectively) (table 4).

We subsequently checked for statistically significant correlations within poorly differentiated and moderately differentiated tumors. In G2 tumors, MCs correlated with hormone receptor expression. ER expression influenced MCit (r_s= 0.309, p= 0.015) as well as MCpt (r_s= 0.346, p= 0.006). PR expression only influenced MCpt density (r_s= 0.357, p= 0.005). Moreover, MCit density

was dependent on MCpt density (r_s= 0.493, p= 0.00005). Both MCs populations were dependent on patients' age (r_s= -0.259, p= 0.044 for MCit and r_s= -0.281, p= 0.028 for MCpt).

Table 4. **Statistically significant correlations established between hormone receptors and mast cells**

Luminal A subtype	Luminal B subtype	TNBC
MCit and PR (r_s= -0.703, p= 0.005)	MCit and MCpt (r_s= 0.590, p= 0.00001)	MCit and MCpt (r_s= 0.687, p= 0.002)
	MCpt and ER (r_s= 0.322, p= 0.029)	
	MCpt and PR (r_s= 0.308, p= 0.037)	
	MCit and ER (r_s= 0.350, p= 0.017)	

***Note**: r_s – Spearman's correlation coefficient, MCit – tryptase+ intratumoral mast cells, MCpt – tryptase+ peritumoral mast cells. The values were considered statistically significant at $p \leq 0.05$.*

In the group of poorly differentiated tumors MCit correlated with the expression of both hormone receptors (ER: r_s= 0.626, p= 0.005 and PR: r_s= 0.561, p= 0.015). MCpt were influenced only by ER (r_s= 0.497, p= 0.036). MCs from the peritumoral area and the intratumoral ones influenced each other (r_s= 0.689, p= 0.002). Statistical analysis of the total cases confirmed the established correlations within the molecular subtypes. MCit correlated statistically significantly with ER (r_s= 0.393, p= 0.0003) and PR (r_s= 0.236, p= 0.035) expression. Similarly, MCpt were dependent on both ER (r_s= 0.378, p= 0.001) and PR (r_s= 0.383, p= 0.0004). In all molecular subtypes included in the given study, peri– and intratumoral MCs were dependent on each other (r_s= 0.445, p= 0.000003).

We further grouped cases according to molecular subtype into luminal and non-luminal ones. To compare the MCs from the luminal and non-luminal groups, we applied the Mann-Whitney U test. It confirmed the influence of hormone receptor expression on the dynamics of both intratumoral MCs (U= 390.5, p= 0.019) and those in the peritumoral areas (U= 361.0, p= 0.008) (table 5).

Table 5. **Mann-Whitney U test results comparing MCs in luminal and non-luminal groups of breast carcinomas**

	MCit	MCpt
Mann-Whitney U	390,500	361,000
Wilcoxon W	600,500	571,000
Z	-2,340	-2,657
p	**,019**	**,008**

***Note:** the luminal group comprised the luminal A, B/ HER2+ and B/ HER2- subtypes; the non-luminal group comprised HER2+ and triple-negative tumors. MCit – tryptase+ intratumoral mast cells, MCpt – tryptase+ peritumoral mast cells*

3.5. S100-positive dendritic cells in breast carcinomas

S100-positive cells were present in most cases (71 out of 75 tumors, 94.6%), but were morphologically varied, which allowed us to group them into several categories. Thus, the first group was made up of S100 positive cells that showed a typical DC morphology. They were medium in size, with an evenly stained cytoplasm, staining intensity ranging from weak to strong. Numerous thin and long, often branched, cytoplasmic processes started from the cell body. Another group of

S100 positive cells showed characteristics similar to those described above, with some differences. These cells also had a stellate shape, but were larger in size with an intensely stained cytoplasm, and the cytoplasmic processes were thick and short. Such DCs were located in the peritumoral regions, and in the inflammatory infiltrate. This aspect was also characteristic of DCs within several lymph nodes analyzed for comparison. The third phenotype of S100-positive cells was completely different from those previously described, consisting of small cells with an intensely colored cytoplasm, lacking cytoplasmic processes.

The next group of S100 positive cells that we observed is of particular interest because it comprised actual tumor cells. Thus, in some tumors the S100 marker could be expressed by all the neoplastic cells within a group of tumor cells (figure 4). In other cases, the tumor islets expressed the protein only regionally. Within the islet, the intensity of the reaction was also heterogeneous, varying from weak to intensely positive. S100-positive cancer cells were characterized by a foamy, unevenly stained cytoplasm with intra-cytoplasmic granulation.

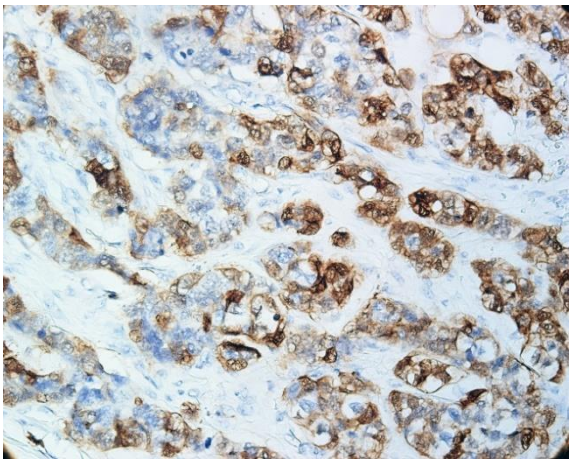

Figure 4. **Invasive ductal carcinoma, NST type. S100 positive tumor areas (40× ob.). Immunoreaction for S100, DAB.**

The last group we observed included cases with basal S100 protein expression. The cells in this category were cuboidal, being arranged in a continuous or discontinuous layer, and the expression of the S100 marker was nuclear and cytoplasmic. It should be noted that all carcinomas with basal S100 protein expression were of the medullary type.

Within some tissue fragments, combinations of the above-mentioned phenotypes could be found, for example both punctate and stellate cells; cancerous S100-positive cells and S100-positive cells within the inflammatory infiltrate (Figure 5).

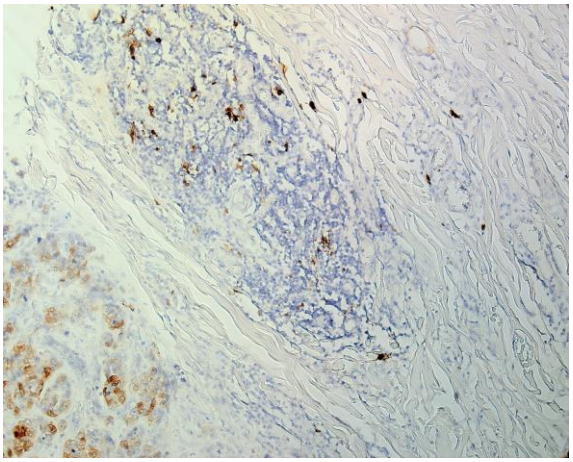

Figure 5. **Invasive ductal carcinoma, NST type. S100-positive tumor islets and DCs from the inflammatory infiltrate (40× ob.). Immunoreaction for S100, DAB.**

Most intratumoral S100-positive cells were recorded in G3 and HER2+ carcinomas. The peritumoral ones were more numerous in the case of G2 and TNBC neoplasms (figure 6).

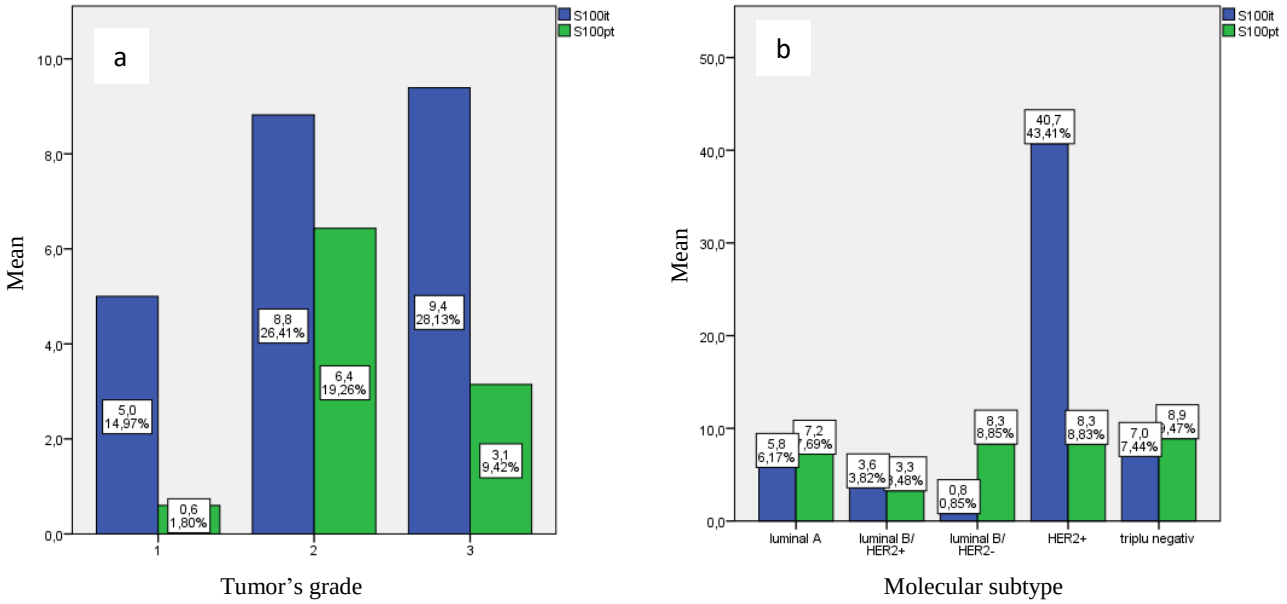

Figure 6. **Distribution of S100+ cells depending on (a) the tumor's grade and (b) molecular subtype.**

S100it were not present in 25 cases (33.3%), of which 17 (68%) expressed the HER2 protein. S100pt were absent in 10 cases (13.3%), of which 7 expressed the HER2 protein (70%). In the case of luminal B-type tumors, S100it cells correlated inversely with age (r_s= -0.357, p= 0.024) and directly with PR expression (r_s= 0.364, p= 0.021). In TNBC tumors, S100it were influenced by tumor grade (r_s= -0.719, p= 0.013). Statistical analysis of all cases revealed statistically significant correlations between S100it and patients' age (r_s= -0.271, p= 0.019), as well as between S100pt and PR expression (r_s= -0.239, p= 0.039). Also, the densities of S100it and S100pt cells correlated with each other (r_s= 0.349, p= 0.002). It should be noted that the correlations established following the analysis of the total cases were also confirmed in G2 tumors. In poorly differentiated tumors statistically significant correlations were not established.

Subsequently, we analyzed S100 protein expression in luminal *versus* non-luminal carcinomas. For this purpose, we performed the Mann-Whitney U test (table 6). The obtained data confirmed that there are differences in S100it density in tumors from the luminal and non-luminal groups (U= 346.0, p= 0,013).

Table 6. **Mann-Whitney U test data for differential expression of S100-positive marker in luminal *versus* non-luminal breast carcinomas**

	S100it	**S100pt**
Mann-Whitney U	346,000	435,000
Wilcoxon W	1886,000	1975,000
Z	-2,492	-1,383
p	**,013**	,167

Note: *the luminal group comprised luminal A, B/ HER2+ and B/ HER2- subtypes; the non-luminal group comprised HER2+ and triple-negative tumors. S100it – intratumoral S100+ cells, S100pt – peritumoral S100+ cells.*

3.6. CD34-positive blood vessels in breast carcinomas

CD34 positive blood vessels were present in both areas studied: intra- and peritmoral, being morphologically different. Thus, 2 types of blood vessels were detected: with lumen and without lumen. More frequently, vessels without a lumen were located intratumorally. Morphologically, they were small, without a lumen, representing cords of endothelial cells (figure 7a). Vessels with lumen were preferentially concentrated at the periphery of the tumor, at the boundaries of the area of invasion and in the peritumoral stroma (figure 7b).

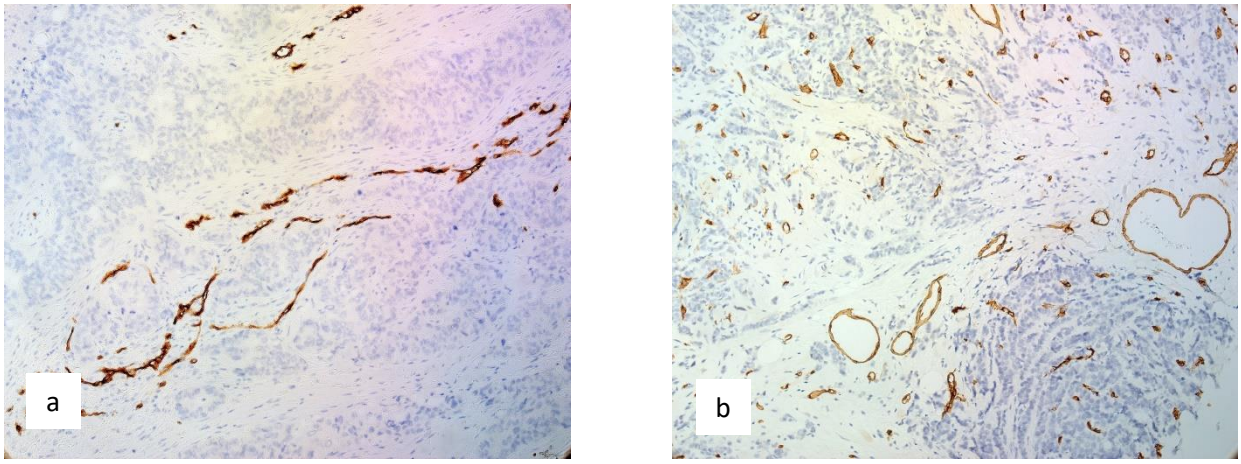

Figure 7. **Invasive ductal carcinoma, type NST. Blood vessels with: (a) intra- and (b) peritumoral location (20× ob.). Immunoreaction for CD34, DAB.**

Peritumoral CD34-positive vessels numerically predominated in poorly differentiated tumors. In the case of moderately differentiated tumors – intra- and peritumoral vessel densities were approximately identical. MVD was also different depending on the molecular subtype. Thus, the density of peritumoral vessels was higher in all molecular subtypes, except for luminal A. The lowest intratumoral MVD values were determined in luminal B/ HER2- subtype.

Analyzing the molecular subtypes separately, no statistically significant correlations were established between MVD, hormone receptor expression, HER2 protein and other parameters included in the study. Analyzing overall cases, the only statistically significant correlation was between CD34it and molecular subtype (r_S= -0.225, p= 0.045). At the same time, we must mention that no statistically significant correlations were established between CD34it or CD34pt and the expression of hormone receptors, HER2 protein or with the other clinicopathological parameters included in this study (age of patients, tumor grade). Comparison of groups according to the luminal/non-luminal criterion did not confirm that MVD is influenced by molecular subtype (p= 0.172 for CD34it and p= 0.619 for CD34pt) (table 7).

Table 7. **Mann-Whitney U test for MVD in luminal *versus* non-luminal group carcinomas**

	CD34it	**CD34pt**
Mann-Whitney U	494,500	574,000
Wilcoxon W	725,500	2344,000
Z	-1,367	-,498
p	**,172**	**,619**

Note: *the luminal group comprised luminal A, B/ HER2+ and B/ HER2- subtypes; the non-luminal group comprised HER2+ and triple-negative tumors. CD34it – blood vessels with intratumoral location, CD34pt – blood vessels with peritumoral location.*

3.7. D2-40 positive lymphatic vessels in breast carcinomas

Immunoreaction for D2-40 was successful in 87 cases, for one of which it was impossible to determine the molecular subtype. Most of the investigated cases (56 tumors, 65.11%) did not contain intratumoral lymphatic vessels. In the other cases, when present, the intratumoral lymphatic vessels were small in size, with a tortuous course, thin wall, often collapsed or had a small lumen. The lumen of the vessels was often septated, and cords of endothelial cells could be observed at the edges, possibly reflecting the process of lymphangiogenesis. Occasionally they contained emboli of tumor cells. In the peritumoral region, lymphatic vessels were absent in 38 (44.18%) cases. It is noteworthy that the majority of these tumors (25 cases) expressed HER2 protein, 16 being of luminal B/HER2+ subtype and 9 belonging to the HER2+ subtupe. In the rest of the tumors, in which the peritumoral lymphatic vessels were present, they had larger dimensions, and a lumen (figure 8).

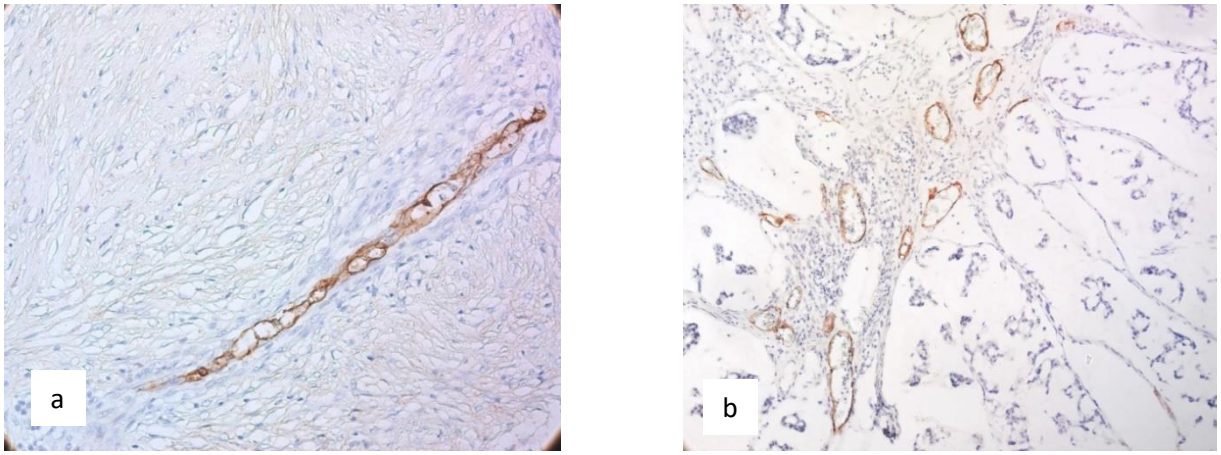

Figure 8. **Invasive ductal carcinomas, NST type. D2-40 positive (a) intra- and (b) peritumoral lymphatic vessels (a - 40× ob., b - 20× ob.). Immunoreaction for D2-40, DAB.**

Peritumoral lymphatic vessels were more numerous than intratumoral ones in all molecular subtypes except the HER2+ one (figure 9a). Of note, the maximum numerical values of both D2-40it and D2-40pt were obtained in TNBC breast carcinomas (19 and 17, respectively). In relation to the histological grade, we determined that in both poorly differentiated and moderately differentiated tumors, peritumoral lymphatic vessels predominate (figure 9b).

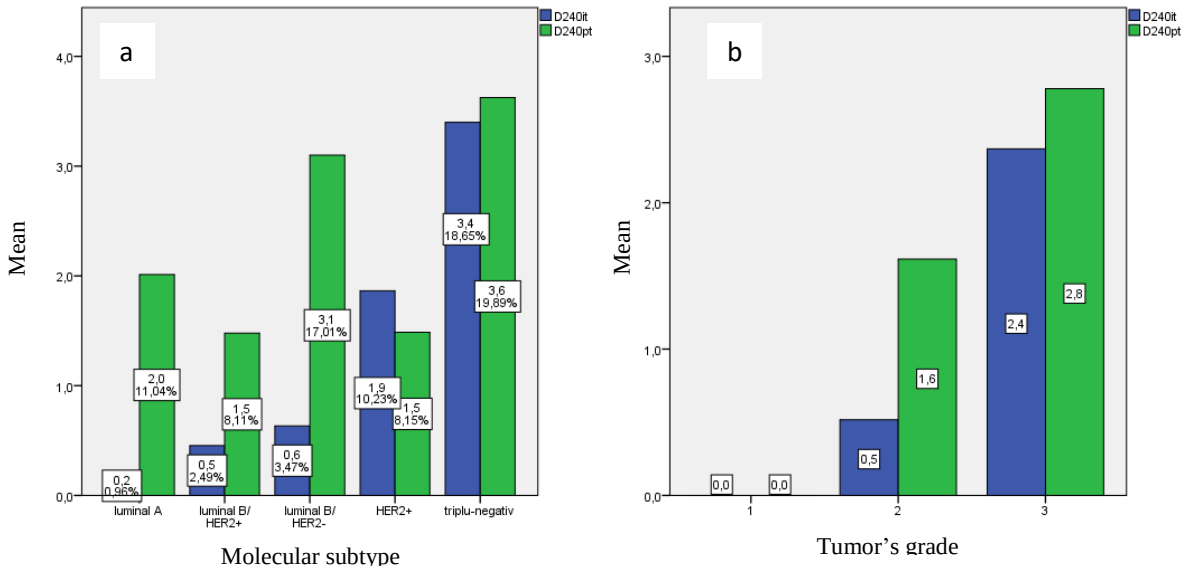

Figure 9. **Lymphovascular density (LVD) based on (a) the molecular subtype and (b) tumor's grade.**

In the case of luminal A subtype, a positive correlation was detected between intratumoral lymphatic vessels and ER expression (r_s= 0.659, p= 0.005). Also within this molecular subtype, we determined that peritumoral LVD increases with the regression of the tumor's grade (r_s= 0.543, p= 0.03). The density of intratumoral and peritumoral lymphatic vessels were interdependent in the HER2+ molecular subtype (r_s= 0.545, p= 0.044). In the group of TNBC tumors, an inverse association was established between peritumoral lymphatic vessels and age (r_s= -0.832, p= 0.001) and a direct association between D2-40it and tumor grade (r_s= 0.657, p= 0.02).

The statistical analysis of the cases according to the luminal and non-luminal groups did not reveal statistically significant correlations between the density of intra- and peritumoral lymphatic vessels and belonging to a certain molecular subtype (p= 0.185 and p= 0.806 for D2-40it and D2-40pt, respectively) (table 8).

Table 8. **Mann-Whitney U test data for LVD in luminal and non-luminal breast carcinomas**

	D2-40it	**D2-40pt**
Mann-Whitney U	660,000	755,000
Wilcoxon W	2490,000	1106,000
Z	-1,327	-,246
p	**,185**	**,806**

Note: *the luminal group comprised luminal A, B/ HER2+ and B/ HER2- subtypes; the non-luminal group comprised HER2+ and triple-negative tumors. D2-40it – lymphatic vessels with intratumoral location, D2-40pt – lymphatic vessels with peritumoral location.*

When analyzing overall cases, we determined more statistically true correlations. Thus, intratumoral lymphatic vessels correlated positively with tumor grade (r_s= 0.269, p = 0.012). A positive correlation was also determined for the association of intratumoral and peritumoral lymphatic vessels (r_s= 0.325, p = 0.002). The observations regarding the expression of the HER2 protein and the lack of peritumoral lymphatic vessels did not find statistical confirmation (p= 0.798).

3.8. Statistical interrelationships between blood vessels, lymphatics and cellular elements included in the study depending on the molecular subtype of the tumor

Analysis of overall cases. The density of blood vessels in the intratumoral stroma was dependent on the number of both intratumoral MCs (r_s= 0.384, p= 0.001) and peritumoral MCs (r_s= 0.232, p= 0.041); but was inversely dependent on peritumoral macrophages (r_s= -0.260, p= 0.021). Peritumoral blood vessels correlated with peritumoral MCs (r_s= 0.349, p= 0.002) but also with lymphatic vessels in the same area (r_s= 0.261, p= 0.033).

Lymphatic vessels in intra- and peritumoral areas showed positive associations (rs = 0.303, p = 0.009). At the same time, the peritumoral vessels were influenced by MCs from the homologous regions (r_s= 0.309, p= 0.010).

Positive associations were also established between intra- and peritumoral S100 positive cell populations (r_s= 0.349, p= 0.002), as well as between MCs from both locations (r_s= 0.506, p= 0.0000014). Peritumoral S100 positive cells correlated with macrophages of the same location (r_s= 0.258, p= 0.030). Later we analyzed the interrelationships within the separate molecular forms, each presenting certain particularities.

Luminal A subtype. Peritumoral macrophages correlated inversely with intratumoral DCs (r_s= -0.540, p= 0.046). Peritumoral LVD was directly dependent on the number of peritumoral MCs (r_s= 0.737, p= 0.006) and positively correlated with the density of peritumoral blood vessels (r_s= 0.789, p= 0.002).

Luminal B subtype. Within this molecular subtype, blood vessel density came to the fore. Thus, the density of peritumoral blood vessels was directly dependent on the expression of the S100 marker (r_s= 0.342, p= 0.035) and the number of peritumoral MCs (r_s= 0.358, p= 0.02). Starting from the idea that MCs stimulates angiogenesis, we checked whether the same correlations also exist in the intratumoral region. The answer was affirmative (r_s= 0.468, p= 0.002 for the association of MCit and CD34it). The idea was also supported by the statistically veridical correlation between the 2 MCs populations (ie intra- and peritumoral): r_s= 0.590, p= 0.0000161. We can thus assume that MCs in the intra- and peritumoral stroma stimulate each other and induce angiogenesis in the respective regions.

HER2+ subtype. We demonstrated the dependence between MCs and LVD in this molecular subtype too, but only in the intratumoral stroma (r_s= 0.872, p= 0.005). Both MCs and lymphatic vessels in this area correlated with macrophages in peritumoral areas (r_s= 0.815, p= 0.007 and r_s= 0.860, p= 0.006, respectively). Lymphatic vessels in the peritumoral stroma were associated with S100 expression in the same area (r_s= 0.727, p= 0.041), and S100 expression in the intratumoral region correlated with peritumoral MCs (r_s= 0.728, p= 0.026).

The triple-negative subtype. LVD in the intratumoral area was influenced not by MCs, but by intratumoral macrophages (r_s= 0.686, p= 0.02). MCs from the same area instead showed more statistically significant correlations, influencing the density of peritumoral blood vessels (r_s= 0.582, p= 0.047) and its counterparts in the peritumoral areas (r_s= 0.668, p= 0.018). Also, an inverse association with peritumoral macrophages was established (r_s= -0.623, p= 0.041).

Figure 10 shows comparative images of some immunoreactions carried out in the study.

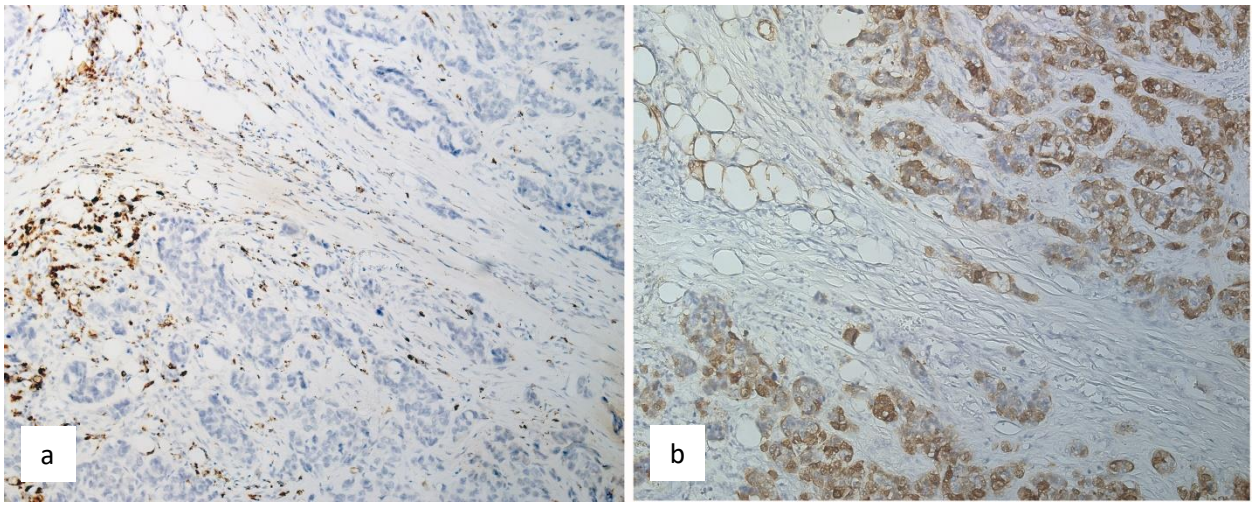

Figure 10. **Invasive ductal carcinoma, type NST. (a) Peritumoral CD68+ cells *versus* (b) intratumoral S100+ cells (40× ob.). Images from the same area of the tumor. Immunoreaction for (a) CD68 and (b) for S100, DAB.**

4. EVALUATION OF STROMAL CHANGES IN BREAST CANCER MOLECULAR VARIANTS

The tumor microenvironment is considered an important factor of tumor progression and response to treatment. As a result, there is increasing interest in the development of new therapies that target the tumor microenvironment, particularly regarding invasive and metastatic progression. The genomic stability of stromal cells makes the development of chemoresistance less likely. In addition, the epigenetic changes that contribute to phenotypic changes, although inherited, are reversible, and there is increasing interest in „normalizing" the altered stroma, thereby abrogating its tumor-supporting role [7]. According to literature data, ER, PR, HER2 and nuclear protein Ki-67 are the main biomarkers needed in the evaluation of the prognosis of breast carcinomas. The positivity of neoplastic cells for ER and/or PR is an important factor for the preliminary evaluation of hormone therapy. HER2 overexpression indicates a higher degree of malignancy, with rapid progression, high tumor's grade, higher probability of recurrence and metastasis [8]. During the evolution of a tumor, a complex and dynamic communication is established between neoplastic cells and cells in the tumor microenvironment; therefore, elucidating the action of stroma components on the expression of the markers included in the current study (ER, PR, HER2) could contribute to understanding the mechanisms of tumor initiation and progression.

One of the actors of the tumor microenvironment addressed in this study is macrophages. The results showed us that the density of these cells differs depending on belonging to the luminal or non-luminal group, the statistical correlation with hormone receptors being an inverse one, that is, peritumoral macrophages could decrease the expression of ER and PR in tumor cells. Also, the less differentiated the tumor, the more intratumoral macrophages there will be. The same data were obtained by Lindsten et al., who stated that the increase of macrophages was negatively associated with ERα or PR, while a positive association was observed with Ki-67. Similar to our results, the association between macrophage infiltrates and HER2 status was inconsistent [9]. Jeong et al. claim that high number of macrophages (CD68+, CD11c+ or CD163+) was associated with high histological grade, high Ki-67 proliferation index, estrogen and progesterone receptor negativity [10]. These data highlight the pro-tumor role of macrophages in peritumoral regions. Being outside the neoplastic area, they inhibit the expression of hormone receptors and can direct the tumor towards a non-luminal phenotype, which cannot benefit from antihormonal therapy and has a more reserved prognosis. In our study, intratumoral macrophages positively correlated with the tumor's grade, confirming that their density increases with the increasing of tumor severity.

Klingen et al. state that high levels of CD163-positive macrophages were associated with blood vessel invasion and lymphatic involvement. In addition, the presence of high levels of CD163+ macrophages was observed more frequently in HER2-positive, basal-like breast carcinomas and TNBC [11]. In our study we also determined statistically significant positive correlations between the density of peritumoral macrophages and CD34it as well as intratumoral MCs, which can stimulate lymphangiogenesis. Globa et al. reported similar data in the case of prostate carcinomas, namely a statistically significant correlation was obtained between the densities of peritumoral CD68+ cells and intratumoral CD34+ vessels [12]. Of note, in our study macrophages correlated with LVD specifically in non-luminal tumors (HER2+ and TNBC). Thus, we can assume that macrophages contribute to the reserved prognosis of non-luminal tumors by stimulating angio- and lymphangiogenesis. A positive correlation between TAM density and increased vascularity, as well

as a reserved prognosis, was reported by Takeya et al. in non-small cell lung cancer [13]. Klingen et al. also argues that tumor-associated macrophages are closely related to vascular invasion and non-luminal subtypes [11].

Another correlation established in our study was that between macrophages and DCs. We believe that this is a natural association, because DC participate together with macrophages in immune reactions, and it makes sense that the densities of these cell populations are interdependent. The associations showed some variation: CD68pt and S100it densities were inversely correlated in luminal A subtype, and CD68pt density was directly correlated with S100pt density in the case-all analysis. Similar to our data, in prostate carcinomas, Globa T. et al. obtained statistically significant correlations both for CD68+ cells (p=0.008 intratumoral, p=0.001 peritumoral), and for S100+ cells (p= 0.036 intraepithelial, p= 0.042 stromal). In addition, the authors described a statistically significant positive correlation between the density of intraepithelial S100+ cells and intratumoral CD68+ cells. Also, the density of S100+ and CD68+ cells was statistically significantly associated with the histological grade of the tumor, which allowed the authors to consider these cells as predictive biomarkers in prostate carcinomas [14].

In our study, correlations were dependent on tumor molecular subtype, and most of them targeted S100it. Thus, S100it correlated inversely with the tumor's grade in the TNBC subtype, and in the luminal B subtype – with PR expression by the neoplastic cells and with the age of the patients. These statistical associations were valid for G2 tumors but not for poorly differentiated tumors. Therefore, with tumor progression the interdependence between S100, patient age and PR expression by tumor cells is lost.

Our observations regarding the link between S100 and HER2 protein expression have been confirmed by other researchers. Thus, Nava et al. hypothesized that S100 family proteins could influence EGF-induced tumor cell growth and metastasis, indirectly contribute to herceptin resistance, and serve as a target in the therapy of HER2+ carcinomas [15].

Macrophages and MCs are known accomplices, being regulators of inflammation, immunological response and angiogenesis in the tumor microenvironment. Our data emphasize the spatial correlations between the 2 cell types. Thus, in non-luminal tumors (HER2+ and TNBC), peritumoral macrophages influenced intratumoral MCs density. This allows us to speculate that initially macrophages are positioned around tumor areas where, by various mechanisms, they attract other cells, such as MCs. MCs in turn exert protumoral effects (angio- and lymphangiogenesis, ECM degradation, etc.) with all the respective consequences. This could be one of the explanations for the aggressiveness of non-luminal tumors. Tamma et al. confirmed the important role of macrophages and MCs in tumor progression and angiogenesis in invasive ductal breast carcinomas [16]. In our previous studies, we showed that MCs dynamics are strongly influenced by hormone receptors and HER2 status. MCit increased in aggressive G3 tumors and can be considered as an unfavorable prognostic factor [17].

On the other hand, a number of clinical studies support the protective role of MCs infiltrating mammary tumors. For example, after a multivariate analysis, Dabiri Sh. et al. found that the presence of stromal MCs is a positive prognostic factor, showing a strong correlation with a high survival rate [18].

Several researchers have observed a high density of MCs in the luminal subtypes of breast carcinoma, which can be treated with hormones and have a more favorable prognosis. This suggests that MCs are associated with less aggressive tumors. On the other hand, we know that estrogens act

as a proliferative factor and can stimulate the development of breast carcinomas [19]. This may explain why, in our study, the density of both MCit and MCpt correlated with ER expression in G2 and G3 tumors. The positive correlations between ER and MCs suggest that estrogens might be chemotactic molecules for MCs. We were particularly interested in the correlation between ER+ carcinoma cells and intratumoral MCs, as some studies have demonstrated that estrogens activate MCs in ovarian endometriosis and that human uterine MCs express ERβ [20]. Keser S. et al. also report correlations between MCit and ER positivity [21]. Rao et al. argue that the latter play a pro-tumorigenic role in bladder cancer by stimulating ERβ and in a murine model of bladder cancer it was shown that a selective ERβ antagonist can inhibit MC-promoted tumor growth [22].

Another aspect of our study was that MCs densities in the 2 analyzed locations were associated with each other, the correlation being a strong one (rs> 0.41). Similar data were obtained by Keser S. et al. They showed that intratumoral MC content correlated positively with peritumoral MC, which in turn correlated with MC in unaffected tissues [21].

There are studies that showed a negative correlation between MCs populations and the tumor's grade, data that were not confirmed by us. In the breast carcinoma study by Sang et al., MC density in patients with Ki-67≤ 14% was significantly higher than MCs content in patients with Ki-67> 14%. Although they could not conclude that MCs are an inhibitory factor of cancer cell proliferation, the results showed that MCs density is also inversely associated with the degree of tumor proliferation, again suggesting that MCs might have an inhibitory effect on cell division [8]. Globa T. et al. disprove this idea. The authors claim that in prostate carcinomas, MCs chymase+ in peritumoral areas and CD117+ MCs in intratumoral areas correlate directly with tumor grade [24]. Some authors have concluded that the prognosis is more unfavorable in the case of increased MCs density in the tumor stroma [8]. Xiang et al. observed more peritumoral MCs in G3 breast cancers, with elevated tryptase being associated with poorly differentiated tumors and more nodal metastases compared with G1 and G2 tumors. The authors also noted that tryptase facilitates cancer cell invasion and migration by activating MMP-2 [25]. Raica et al. revealed strong positive correlations between MC populations and lymphatic vessels in some molecular subtypes of breast cancer, thus supporting the idea of MCs involvement in metastasis through lymphangiogenesis [26]. In our study, peritumoral MCs correlated with LVD in luminal A subtype. In the HER2+ subtype, MCs in the intratumoral area influenced by intratumoral LVD.

Keser S. et al. demonstrated that there are interrelationships between MCs density and LVD [21]. Similar to our data, several studies report that MCs were generally observed in the stroma adjacent to neoplastic cells and near vascular structures [18, 27]. Angio- and lymphangiogenesis allow tumor progression from the *in situ* to the invasive form, with the initiation of metastasis. Blood and lymphatic vessels, however, provide diametrically opposed conditions for tumor cell migration and survival. These conditions are closely related to the distinct functions and structural features of these two systems (eg difference in lumen, BM structure, anchorage to ECM, etc.) [28].

Lymphangiogenesis is essential for the escape of cancer cells from their primary site to regional lymph nodes, resulting in systemic tumor spread. In our study, in both luminal and non-luminal molecular subtypes, microvascular density and LVD were dependent on MCs. Of note, in the luminal subtypes, peritumoral MCs influenced lymphatics and blood vessels in the same area. In non-luminal subtypes, intratumoral MCs density correlated with intratumoral vessels. This may be one of the explanations for the higher aggressiveness of non-luminal carcinomas.

Also, in the specialized literature, there are data that during breast cancer progression, MCs can contribute to stromal remodeling and myofibroblast differentiation, through tryptase released in the ECM. In prostate carcinomas, Globa T. et al. determined a total correlation between CD34pt and tryptase in peritumoral areas as well as the absence of a significant association between them in intratumoral areas [29]. In lesions of the uterine cervix, Cebanu A. et al. assumed that MCs have a lymphangiogenic role. Their results indicated a significant correlation between the number of lymphatic vessels and MCs in peritumoral areas in invasive carcinomas, compared to squamous metaplasia and carcinoma *in situ* [30].

DC exert a strong pro-angiogenic activity that is mediated by the angiogenic growth factor VEGF-A. DCs can also transdifferentiate into endotheliocytes, thus contributing to angiogenesis and vasculogenesis. This aspect may explain the results of our study, namely the interrelationships between CD34 and S100 expression. Similar to breast carcinomas, Yamagata et al. established that in oral squamous cell carcinomas, CD163-positive macrophages promote lymphangiogenesis by expressing VEGF-C, which contributes to regional lymph node metastasis [31]. Thus, angiogenic mediators, produced by various stromal cells and by cancer cells themselves, can have a paracrine action, creating a vicious circle. Sammarco G. et al. confirms that also in gastric cancer MCs density is increased and there is a correlation with angiogenesis, the number of metastases in the lymph nodes and the survival of these patients. MCs exerts a protumorigenic role in gastric cancer by releasing angiogenic (VEGF-A, CXCL8, MMP-9) and lymphangiogenic (VEGF-C and VEGF-F) factors [32].

In our study, by using the D2-40 antibody we demonstrated the existence of lymphatic vessels inside the tumor. In the given study we demonstrated the interrelationship between D2-40it and D2-40pt, the correlation being moderate (rs= 0.325). The higher density of peritumoral lymphatic vessels compared to intratumoral ones may suggest that they form a network at the periphery of the tumor and are functional vessels. In our study we determined a statistically significant correlation between intratumoral lymphatic vessel density and ER expression by tumor cells in the luminal A subtype, implying a hormonal control of the lymphatic system. Despite numerous data indicating a beneficial effect of estradiol on the blood vessels, surprisingly little data is known about its effects on the lymphatic endothelium. We know that the influence of estradiol on the lymphatic endothelium is exclusively mediated by the ERα receptor. This is a major difference from the endothelium of blood vessels, which expresses both ERα and ERβ [33]. Starting from these premises, Morfoisse F. et al. modeled lymphedema in mice and concluded that ERα directly regulates lymphangiogenic genes, which stimulates the migration and sprouting of lymphatic endothelium. This group of researchers also established that blocking ERα using a selective estrogen modulator such as tamoxifen has a detrimental effect on lymphatic drainage and vessel function, increasing the risk of developing secondary lymphedema [34], which also occurs in the treatment of luminal breast cancer.

The statistically significant correlations between CD34 and D2-40 we established suggest the close connection between these processes. Both *in vivo* animal models and *in vitro* assays have indicated that lymphangiogenesis occurs after angiogenesis. Therefore, the formation of lymphatic vessels may rely not only on lymphangiogenic factors but also on angiogenic factors [28]. In cervical lesions, Şaptefrați et al. observed that the main source of VEGF is tumor cells. VEGF was not expressed in normal cervical specimens. The marker was identified in a small number of CIN1 cases, but VEGF expression intensified as the cervical neoplasia progressed. The data of these authors attest to the acquisition of the angiogenic phenotype of cervical lesions at the stages of CIN2-3, that is, much earlier than the appearance of the actual carcinoma. Immunohistochemical expression of VEGF

was not only limited to epithelial cells, but was also observed in stromal cells, which in cases with invasive carcinomas expressed VEGF in a proportion of over 60%.

The stromal microenvironment plays a major role in maintaining normal tissue homeostasis and promoting tumor growth. Increasing evidence suggests that the normal tissue microenvironment is a barrier to tumorigenesis, while aberrant proinflammatory signals destabilize tissue homeostasis and promote tumorigenesis. Thus, cancer cells educate the cells in the tumor microenvironment and force them to play by their own rules. In this paper, key players in the stroma of breast tumors and their role in tumorigenesis and cancer progression were analyzed. However, a key question remains: what comes first, the dysfunction of epithelial cells or the dysfunction of their microenvironment?

GENERAL CONCLUSIONS

1. The tumor microenvironment is an essential element in the structure of breast carcinomas, influencing their growth and progression. The interrelationships between epithelial cells and stromal structures are complex, stimulatory or inhibitory, thus management of the stroma is imperative to potentiate or slow the expansion of neoplastic cells.
2. In luminal breast carcinomas, the density of mast cells in the peritumoral areas prevails over that of macrophages and dendritic cells showing a statistically significant positive correlation. In ER+ and PR+ tumors, the intratumoral areas showed a higher density of mast cells compared to the peritumoral ones, thus hormone-positive tumors show chemoattractiveness for tryptase+ cells, stimulating their migration.
3. Most hormone-independent breast carcinomas showed a high density of macrophages in both intratumoral and peritumoral stroma. In ER+ and PR+ carcinomas the density of CD68+ cells registered low values.
4. Loss of estrogen/progesterone receptors is accompanied by increased density of peritumoral S100+ cells. S100 positivity of cancer cells suggests their antigenic modulation, with property transfer and „mimicry” or may be the consequence of fusion of the dendritic cell with the neoplastic cell.
5. Mast cells are the cellular elements capable of modulating the stroma of breast carcinomas, being involved in angiogenesis and lymphangiogenesis, the high density of mast cells being accompanied by the decrease in the number of dendritic cells and macrophages.
6. In poorly differentiated breast carcinomas, the density of blood and lymphatic vessels is higher, with predilection in peritumoral areas, thus creating favorable conditions for tumor expansion. Breast carcinomas expressing receptors for estrogen, progesterone or HER2 protein do not show statistically significant correlation with vascular density.

RECOMMENDATIONS

1. In the study of stromal elements, we propose to determine their density separately in the intra- and peritumoral areas.
2. In the study of cell varieties, it is recommended to use the median as a threshold value to further divide the data into 2 categories: low density and high density.
3. Determination of CD68+ macrophages and S100+ dendritic cells in neoplastic mammary gland specimens allows selection of patients who could benefit from immune therapy.

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- **Articles in scientific journals abroad:**

✓ **articles in ISI, SCOPUS and other international databases**

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2. **Carpenco E**. Tumor associated macrophages in breast cancer. In: *Moldovan Medical Journal*, 2019, 62(1):24-27. ISSN 2537-6373. https://ibn.idsi.md/ro/vizualizare_articol/75800
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10. **Carpenco E.** Lymphatic vessels density and its implications in breast cancer. *In: MedEspera 2020: abstract book. The 8th International Medical Congress for Students and Young Doctors*, Chişinău, 24-26 septembrie 2020, pp. 244-245.

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15. **Carpenco E.,** David V., Foca E. Mastocitele în cancerele mamare luminale *versus* non-luminale. *Conferința științifică anuală Cercetarea în biomedicină și sănătate. Calitate, excelență și performanță. Zilele Universităţii de Stat de Medicină și Farmacie „Nicolae Testemitanu" din Republica Moldova*. Chișinău, 20-22 octombrie 2021.

- **Participation with posters at scientific forums:**

16. **Carpenco E.** Macrofagele asociate tumorilor în cancerul mamar. *Poster moderat la Zilele Universităţii de Stat de Medicină și Farmacie „Nicolae Testemitanu" din Republica Moldova,* Chișinău, 15-18 octombrie 2019.

ADNOTARE

Carpenco Ecaterina

„Particularități ale stromei în subtipurile moleculare de cancer mamar”

Teză de doctor în științe medicale, Chișinău, 2023

Structura tezei: Teza este expusă pe 92 pagini de text de bază: introducere, 4 capitole, concluzii generale și recomandări practice, 47 figuri și 26 tabele. Bibliografia include 209 surse. Rezultatele studiului au fost publicate în 9 lucrări științifice.

Cuvinte-cheie: ER, PR, HER2, subtipuri moleculare, microambianță, stroma intratumorală, stroma peritumorală.

Scopul lucrării: Studiul varietăților celulare și al elementelor vasculare din stroma carcinoamelor mamare în dependență de subtipul molecular al tumorii în vederea îmbunătățirii diagnosticului și prognosticului cancerului mamar.

Obiectivele lucrării: Evaluarea histologică și imunohistochimică a varietăților celulare (CD68, S100, triptaza mastocitară) din stroma carcinoamelor mamare în diferite subtipuri moleculare ale tumorii. Studiul particularităților rețelelor vasculare sangvine (CD34) și limfatice (D2-40) în carcinoamele mamare în funcție de subtipul molecular. Analiza interelațiilor între elemente celulare, vase sangvine și limfatice *versus* subtipul molecular al tumorii. Elucidarea impactului diverselor elemente stromale asupra diagnosticului, prognosticului și evoluției carcinomului mamar.

Noutatea și originalitatea științifică: Pentru prima dată a fost efectuat un studiu comparativ al profilului molecular al carcinoamelor mamare *versus* elemente celulare (macrofage, mastocite, celule dendritice) și vasculare (sangvine și limfatice) din stroma tumorală. În premieră a fost evaluat gradul expresiei unor markeri celulari (CD68, S100, triptaza, CD34, D2-40) în ariile intra- și peritumorale în raport cu expresia markerilor ER, PR și HER2. Au fost descrise modificările cantitative și calitative ale componentelor stromei tumorale în subtipurile luminale și non-luminale.

Importanța teoretică și aplicativă a lucrării: Evaluarea cantitativă și calitativă a elementelor stromale în carcinoamele mamare permite completarea cunoștințelor existente despre rolul stromei în evoluția și progresarea neoplaziilor mamare, cu accent asupra diferențelor în grupul luminal *versus* non-luminal.

Implementarea rezultatelor științifice: Rezultatele studiului dat au fost implementate în activitatea didactică și științifică a Catedrei de histologie, citologie şi embriologie şi a Laboratorului de morfologie al USMF „Nicolae Testemitanu”.

АННОТАЦИЯ

Карпенко Екатерина

„Особенности стромы в молекулярных подтипах рака молочной железы”

Диссертация на соискание ученой степени доктора медицинских наук, Кишинев, 2023

Структура диссертации: Диссертация представлена на 92 страницах основного текста: введение, 4 главы, общие выводы и практические рекомендации, 47 рисунков и 26 таблиц. Библиография включает 209 источников. Результаты исследования опубликованы в 9 научных статьях.

Ключевые слова: ER, PR, HER2, молекулярные субтипы, микроокружение, строма опухоли, перитуморальная строма.

Область исследования: патологическая анатомия.

Цель исследования: Изучение разновидностей клеток и сосудистых элементов стромы карцином молочной железы в зависимости от молекулярного подтипа опухоли с целью улучшения диагностики и прогноза рака молочной железы.

Задачи исследования: Гистологическая и иммуногистохимическая оценка клеток стромы карцином молочной железы в зависимости от молекулярного подтипа опухоли. Изучение особенностей кровеносной и лимфатической сети в зависимости от молекулярного подтипа карциномы. Анализ взаимосвязей между клетками, кровеносными и лимфатическими сосудами в зависимости от молекулярного подтипа опухоли. Изучение влияния различных стромальных элементов на прогноз карциномы молочной железы.

Новизна и оригинальность исследования. Впервые проведено сравнительное исследование молекулярного профиля карцином молочной железы относительно с клеточными (макрофаги, тучные клетки, дендритные клетки) и сосудистыми (кровяными и лимфатическими) элементами в строме опухоли. Впервые оценена степень экспрессии некоторых клеточных маркеров (CD68, S100, триптазы, CD34, D2-40) в интра - и перитуморальной областях по отношению к экспрессии ER, PR и HER2. Описаны количественные и качественные изменения стромальных компонентов опухоли при люминальном и нелюминальном подтипах.

Практическая значимость: Количественная и качественная оценка стромальных элементов в карциномах молочной железы позволяет дополнить существующие знания о роли стромы в развитии и прогрессировании неоплазий молочной железы с акцентом на различия в люминальной и нелюминальной группе.

Внедрение результатов: Результаты данного исследования были внедрены в дидактическую и научную деятельность Кафедры гистологии, цитологии и эмбриологии и Лаборатории морфологии ГУМФ им. Николае Тестемицану.

ANNOTATION

Carpenco Ecaterina

„Peculiarities of the stroma in the molecular subtypes of breast cancer"

PhD, Thesis in Medical Science, Chisinau, 2023

Thesis structure: The thesis is presented on 92 pages of basic text: introduction, 4 chapters, general conclusions and practical recommendations, 47 figures and 26 tables. The bibliography includes 209 sources. The results of the study were published in 9 scientific papers.

Keywords: ER, PR, HER2, molecular subtypes, microenvironment, tumor stroma, peritumoral stroma.

Field of study: Pathology**.**

Purpose of study: The study of cell varieties and vascular elements in the stroma of breast carcinomas depending on the molecular subtype of the tumor in order to improve the diagnosis and prognosis of breast cancer.

Objectives: Histological and immunohistochemical evaluation of cell varieties (CD68, S100, mast cell tryptase) from the stroma of breast carcinomas in different molecular subtypes of tumors. The study of the peculiarities of the blood vascular (CD34) and lymphatic (D2-40) networks in breast carcinomas according to the molecular subtype. Analysis of interrelationships between cellular elements, blood vessels and lymphatics *versus* tumor molecular subtype. Elucidating the impact of various stromal elements on the diagnosis, prognosis and evolution of breast carcinoma.

Scientific novelty and originality: For the first time, a comparative study of the molecular profile of breast carcinomas *versus* cellular (macrophages, mast cells, dendritic cells) and vascular (blood and lymphatic) elements in the tumor stroma was performed. For the first time, the degree of expression of some cell markers (CD68, S100, tryptase, CD34, D2-40) in intra- and peritumoral areas was evaluated in relation to the expression of ER, PR and HER2 markers. Quantitative and qualitative changes in tumor stromal components in luminal and non-luminal subtypes were described.

The theoretical and applied importance of the work: Quantitative and qualitative assessment of stromal elements in breast carcinomas allows to complement the existing knowledge about the role of stroma in the evolution and progression of breast neoplasias, with emphasis on the differences in the luminal *versus* non-luminal group.

Implementation of the scientific results: The results of the given study were implemented in the didactic and scientific activity of the Department of Histology, cytology and embryology and the Laboratory of morphology of „Nicolae Testemitanu" sUMPh.