Medical University - Pleven

Faculty of Medicine

Department of Pathology

Dr. Polina Damyanova Dimitrova

Morphological and immunohistochemical aspects of the antitumor immune response in different subtypes of breast cancer

ABSTRACT

of a dissertation

to acquire an educational and scientific degree

"Doctor"

Field of higher education: 7. Health care and sports
Professional direction: 7.1. Medicine
Doctoral program: "Pathology and cytopathology"

Research supervisor – Prof. Dr. Savelina Ljubenova Popovska, MD, Ph.D., DSc

Official Reviewers:

Assoc. Prof. Ivan Nedkov Ivanov, MD, PhD

Prof. Dr. Nikolay Dimitrov Hadjiolov, MD, PhD, DSc

Pleven 2022

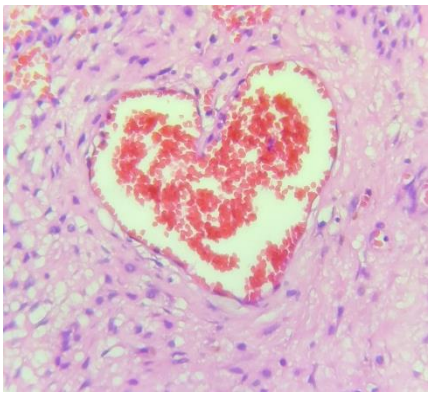
The dissertation is written on 206 standard printed pages. The material is illustrated with 41 figures, 27 tables, 9 microphotographs and 4 appendices.

The bibliography includes 295 titles – 3 in Cyrillic and 292 in Latin.

The author is a full-time doctoral student at the Department of Pathology, Faculty in Medicine, Medical University – Pleven

Note: the numbering of the figures and the tables does not correspond to that in the dissertation.

The dissertation is discussed and set for a public defense of an extended Department Council of the Department of Pathology, Faculty of Medicine, Medical University - Pleven, held on 30.08.2022.



"A true artist is not one who is inspired, but one who inspires others" Salvador Dali

With gratitude to my family and my Research supervisor - for the inspiration, support and patience during my scientific work!

The public defense will take place on Nov 11, 2022, from 1.00 p.m. in Ambroise Pare Hall, MU – Pleven.

Scientific jury composed of:

Chairman: Assoc. Prof. Dr. Ivan Nedkov Ivanov, MD, PhD

Members: Prof. Dr. Nikolay Hadzhiolov, MD, PhD, DSc

Assos. Prof. Dr. Deyan Lyudmilov Dzhenkov, MD, PhD

Assoc. Prof. Dr. Julian Rumenov Ananiev, MD, PhD

Assos. Prof. Dr. Angel Danchev Yordanov, MD, PhD

Reserve members:

Prof. Dr. Veselin Todorov Belovejdov, MD, PhD

Assos. Prof. Dr. Strahil Asenov Strashilov, MD, PhD

The defense materials are published on the website of MU-Pleven (www.mu-pleven.bg) and are available to those interested in the Scientific Department of the Faculty of Medicine, MU Pleven.

ABBREVIATIONS USED IN THE TEXT:

BC - breast cancer
CD - cluster of differentiation
CK - Cytokeratin
CTL - cytotoxic T lymphocytes
CTLA-4 - cytotoxic T-lymphocyte–associated antigen 4
DAB - diaminobenzidine
ECIS - European Cancer Information System
ER - Estrogen receptor
FFPE - formalin fixed paraffin embedded tissue sampleFoxp3 - Forkhead Box Protein 3
G – grade/ degree of differentiation
HE - hematoxylin – eosin
HER2 - Human epidermal growth factor receptor 2
HPF – high power field
HRP - Horseradish Peroxidase
IHC – immunohistochemistry/immunohistochemical mathod
IR - immune response
IS - immune system
ISH - In situ hybridization
IT – intratumoral lymphocytes
LN – lymph node
LPBC - Lymphocyte-predominant breast cancer
LumA – Luminal A breast cancer
LumB – Luminal B breast cancer
LVI - lymphovascular invasion
MW - microwave oven
NST - no special type breast cancer
OS - overall survival
PD-1 - Programmed cell death-1
PD-L1/PD-L2 - Programmed cell death ligand 1/2
PR – progesterone receptor
ST – stromal lymphocytes
Th - T helper lymphocytes
TILs - tumor-infiltrating lymphocytes
TN - triple negative breast cancer
TNM - Tumour, node, metastasis
TP - tumor proportion
Treg - regulatory T lymphocytes
WHO - World Health Organization

I. INTRODUCTION

Currently, there are conflicting data on the role of tumor-infiltrating lymphocytes (TILs) in local antitumor immunity and their significance on prognosis in breast carcinoma (BC) patients. The effect of the interaction of TILs with tumor cells, for the importance of the immune "checkpoint" pathways in the regulation of the immune response (IR), is not fully understood.

The complexity of the problem is due to the heterogeneity of the primary tumor in this neoplasm, the heterogeneous cellular composition of the inflammatory infiltrate, the unequal distribution of immune cells in different areas of the tumor and in individual subtypes of BC. The various BC groups, incl. the defined molecular types - Luminal A and B (LumA and LumB), Basal/triple negative (TN) and Human epidermal growth factor receptor 2 (HER 2) enriched, are characterized by different molecular and genetic alterations, have different prognosis and response to therapy. The basal subtype, expressing basal cell cytokeratins, is associated with the worst prognosis and genetic instability, due to the multiple mutations present in it. On the other hand, the mutation products are perceived by the body as neoantigens inducing IR and this turns these types of tumors into more immunogenic neoplasms, characterized by a more pronounced inflammatory infiltrate in the stroma, in the tumor and in the non-neoplastic tissue. But whether the immune cells present are active with effective antitumor IR or are suppressed as a result of interaction both with each other and with the tumor cells, or due to the involvement of immune inhibitory pathways (e.g. associated with Programmed cell death-1 (PD-1), Programmed cell death ligand 1/2 (PD-L1/PD-L2) and cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) molecules) are facts whose clarification will increase the possibility of desired immune modulation. The role of CTLA-4 and PD-1/PD-L1 involved in inhibitory immune cell pathways and the process of programmed cell death is not well studied in the different subtypes of BC. In recent years, inhibitors targeting these molecules have shown promising results in various types of neoplasms, correlating with their tumor and/or immune expression and the effect of therapy. Reactivation of latent TILs by applying immunotherapy, incl. CTLA-4 and PD-L1/PD-1 inhibitors or specific targeted therapy promises a new strategy in the treatment of breast cancer as well.

II. GOAL AND OBJECTIVES

1. Aim of the research

Based on the literature data on the clinical-epidemiological significance of BC, the heterogeneity of this neoplasm and the controversial role of immune system (IS) in it, we set out to study some morphological and immunohistochemical aspects of the antitumor immune response in different subtypes of breast carcinoma.

2. Tasks

To fulfill the main goal, we set ourselves the following tasks:

2.1. In the selected cases and in the surrogate molecular subtypes of BC:

- to establish the composition of TILs, by determining the expression of CD20, CD3, CD4, CD8 and FoxP3, marking respectively B-, T- and T-lymphocyte subtypes – helper, cytotoxic and regulatory cells;
- to determine the amount of stromal (ST) TILs and intratumoral (IT) and ST TILs subtypes;
- to study the expression of PD-L1 and CTLA-4 in tumor and immune (ST and IT) cells;
- to determine the basal subtype of carcinomas by studying the expression of basal cell cytokeratins - CK5/6 and CK17;
- to approve and validate a methodology for determining in the routine practice of the TILs subtypes - B-, T- and T-helpers, cytotoxic and regulatory, the immune "checkpoint" molecules PD-L1 and CTLA4 and basal phenotype in BC.

2.2. In the studied cases with BC and in the individual subtypes - LumA, LumB, HER2 and TN, to determine the frequency of:

- studied epidemiological [sex, age, 5-year overall survival (OS)] and clinicopathological characteristics [type of surgical intervention, clinical stage, histological degree of differentiation (grade - G), size and histological variant of the tumor, basal subtype, status of axillary lymph nodes (LN), lymphovascular invasion (LVI)];
- studied immune factors (% TILs; IT and ST CD3, CD4, CD8, CD20 and FP3 lymphocytes; % tumor cells positive for PD-L1 and CTLA4; % IT and ST immune cells positive for PD-L1 and CTLA4).

2.3. To look for correlations between the investigated indicators (epidemiological, clinico-pathological and immune) in the whole sample and in individual subtypes of BC.

2.4. To look for differences between BC subtypes in terms of the studied indicators (epidemiological, clinico-pathological and immune).

2.5. To investigate additional aspects of antitumor IR (in the whole sample and/or in different BC subtypes) by determining:

- the preferred localization (intratumoral and/or stromal) of lymphocyte subtypes and PD-L1 and CTLA-4 positive immune cells;
- predominant subtype of lymphocytes – B or T and subtype T;
- immunogenicity of BC subtypes.

2.6. To investigate the prognostic value (in the patients with BC included in the study) of:

- TILs and their subspecies;
- the positivity for the immune "checkpoint" molecules PD-L1 and CTLA4 in tumor and immune cells;
- basal phenotype of carcinomas.

III. MATERIALS AND METHODS

1. Qualitative method - Analysis of archival information arrays with histological data and review of archival tissue samples.

Patients

100 patients with morphologically proven primary invasive BC grouped into four molecular surrogate subtypes (Luminal A and Luminal B-like, HER2-positive and triple negative - TN), 25 cases each, were retrospectively analyzed.

The participants in the study were included on a random basis, from archive lists of the Department of General and Clinical Pathology at University Hospital "G. Stranski" - Pleven, based on a selection of tumors from archival materials, where a sufficient amount of tissue material is available and the study does not in any way threaten its damage and depletion.

Until the previously determined number of patients was collected, the archive documentation and biopsy samples of a total of 290 cases of breast cancer, diagnosed in the period 05.01.2011 - 31.12.2014, were reviewed.

All patients selected for the study had no evidence of inflammatory diseases or those associated with an inflammatory reaction in the breast. Cases with preoperative antitumor therapy, with missing or at risk of exhaustion archival tissue material, were excluded.

Demographic data (sex, age, 5-year overall survival) and studied clinicopathological factors (type of surgical intervention, clinical stage, G, tumor size and histological variant, axillary lymph node status, LVI) were systematized and registered on questionnaire specially designed for the study.

Patients are included in:

1. RESEARCH PROJECT No. 19/2015 - Study on distribution, localization and immunophenotype of tumor infiltrating lymphocytes in different molecular subtypes of breast cancer, MU-Pleven)
2. RESEARCH PROJECT No. 11/2018 - Study on the expression of the immune checkpoint inhibitors PD - L1 and CTLA - 4 in the different molecular subtypes of breast cancer, MU- Pleven
3. RESEARCH PROJECT No. D2/2020 - Study on the expression of BRCA1 and BRCA2 related proteins in different molecular subtypes of breast cancer determined by immunohistochemical method, MU - Pleven)
4. European Regional Development Fund through the: Operational Programme "Science and Education for Smart Growth", with a leading organization MU-Pleven, grant no BG05M2OP001-1.002-0010-C01 (2018-2023)

T he projects have been approved by the ethics committee at the Medical University, Pleven. All patient data were summarized and coded, and tissue materials were examined without the need for identification of the study cases.

Due to insufficient volume and risk of exhaustion (paraffin block with thickness of tissue material <1mm) of archival tissue materials studied in project No. 19/2015, for the implementation of projects No. 11/2018, D2/2020 and BG05M2OP001-1.002-0010, at 26 of the selected cases had new tissue samples selected.

2. Morphological method - Routine examination, allowing diagnosis, classification, grading and staging of tumors based on the determination of tissue and cellular structural characteristics in the studied sample.

2.1. Histological (tissue) examination (from histos - tissue) - conducted mainly for diagnostic purposes.

The tissue taken during a surgical intervention, the so-called biopsy, is subsequently processed in a pathology laboratory where, after fixation (routinely in 10% buffered formalin) and additional procedures, it is embedded in a paraffin block. Trained personnel make tissue sections from it, 2 to 5 microns thick. Mounted on glass slides and after subsequent routine staining (with HE), histological preparations are obtained for observation under a light microscope by a pathologist. He provides a histological result including a description of his observations and a diagnosis based on them.

2.2. Immunohistochemical study

In cases where the initial histological assessment does not allow an accurate diagnosis, incl. assigning the tumor to a certain classification and to determine tumor cell activity, it is recommended to examine the sample with additional methods, incl. IHC. The latter is highly effective and established in routine pathological practice. It is based on a specific antigen-antibody reaction, allowing the identification of specific cellular structural components.

2.3. Histological and IHC examination (in accordance with the recommendations, current to the period of the study) applied in the analysis of the selected cases.

Archival slides with HE staining were evaluated in the examined BC patients. BC cases are subtyped according to the World Health Organization (WHO) histological classification (4th edition). The Nottingham grading system (modified scheme of Elston & Ellis, 1991) was used to grade invasive carcinomas. Tumors were staged according to the 7th revision of the TNM classification of the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) from 2010.

On the 290 reviewed cases, the archival slides with staining for ER, PR, HER2 and Ki 67 were evaluated, after analysis of which the clinical-pathological surrogate molecular subtypes of BC were determined - Luminal A and Luminal

B-like, HER2-positive and TN, according to the international expert consensus for primary treatment of early BC St Gallen 2013– **Table 1.**

Microscopic evaluation of ER, PR, and HER-2 /neu was done according to the American Society of Clinical Oncology/College of American Pathologists Guidelines (ASCO/CAP guidelines). The interpretation of the IHC - Ki67 result was according to the recommendations of the Working Group on BC.

Table 1. *Immunohistochemical method applied to define surrogate molecular subtypes of BC*

Phenotype	Clinicopathological definition
Luminal A-like	ER+,PR+(>/=20%), HER2-,Ki67<14%
Luminal B-like - HER2 negative	ER+, HER2- and at least one: PR-/<20%, Ki67>/=14%
Luminal B-like- HER2 positive	ER+/PR+,ER+/PR-;ER-/PR+, HER2+ (IHC: HER2=3+; ISH+) and any value of Ki67
HER2-positive (non-luminal)	ER-, PR-, HER2+, every % Ki67
Triple negative (TN)	ER-, PR-, HER2-, every % Ki67

After applying the inclusion and exclusion criteria, 100 patients were selected, divided into the four categories indicated, 25 cases in each group.

For each patient participating in the study, one histological slide was selected for microscopic and computer-assisted evaluation (Uthscsa image tool v. 3.00) of the percentage of stromal TIL% in routinely stained (with HE) tissue sections. Based on the recommendations of the International TILs Working Group 2014 - **Figure 1**, the tumors included in the study were subtyped as: BC with TILs >50% (lymphocyte-predominant); BC with available TILs, but ≤50%; "tumors - deserts" in which no stromal TILs are found.

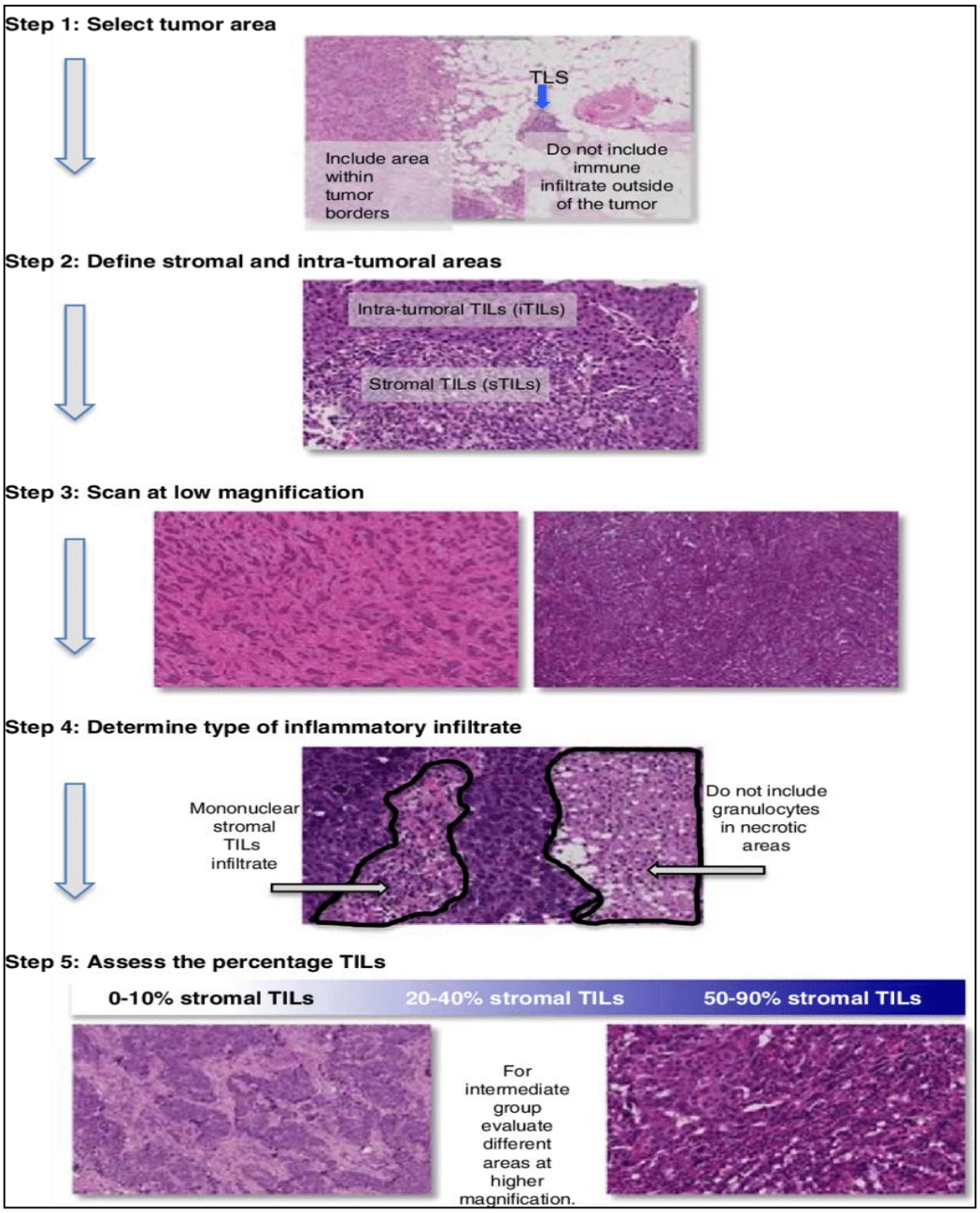


Figure 1. *Standardized approach for histological assessment of %TIL in BC, HE staining*

From the relevant paraffin block of the selected histological number for each patient, slides were prepared for IHC evaluation of TILs subtypes, to determine the expression of immune "checkpoint" molecules - PD-L1 and CTLA4, as well as to report the positivity of basal cytokeratins - CK5/ 6 and CK17 (consumables used - **Table 2**).

Table 2. *Reagent used*

Primary antibody	Clene	Manufacturer	Dilution	Visualizing system	Automated slide processing system	Cellular/molecular specificity
CD3, Rb	Polyclonal	Dako, Agilent, Glostrup, Denmark	RTU	EnVision™ FLEX, High pH, (Link), DAKO	AutostainerLink 48, DAKO	Normal and neoplastic T-lymphocytes
CD4, Mo	4B12, Isotype: IgG1, kappa	Dako, Agilent, Glostrup, Denmark	RTU	EnVision™ FLEX, High pH, (Link), DAKO	AutostainerLink 48, DAKO	Normal T-helper cells
CD8, Mo	C8/144B, Isotype: IgG1, kappa	Dako, Agilent, Glostrup, Denmark	RTU	EnVision™ FLEX, High pH, (Link), DAKO	AutostainerLink 48, DAKO	Normal cytotoxic T cells
CD20, Mo	L26, Isotype: IgG2a, kappa	Dako, Agilent, Glostrup, Denmark	RTU	EnVision™ FLEX, High pH, (Link), DAKO	AutostainerLink 48, DAKO	Normal and neoplastic B-lymphocytes
FoxP3, Mo	236A/E7, Isotype: IgG1, kappa	Bioscience, San Diego, USA	1:100	EnVision™ FLEX, High pH, (Link), DAKO	AutostainerLink 48, DAKO	A subtype of normal regulatory T lymphocytes
PD-L1, Mo	22C3, Isotype: IgG1, kappa	Dako, Agilent, Glostrup, Denmark	1:50	EnVision™ FLEX, High pH, (Link), DAKO + Signal Amplification Reagent - EnVision FLEX+ Mouse (LINKER)	AutostainerLink 48, DAKO	PD-L1 protein expressed on some tumor and immune cells
CTLA-4, Mo	F-8, Isotype: IgG1, kappa	Santa Cruz Biotechnology, USA	1:100	EnVision™ FLEX, High pH, (Link), DAKO + Signal Amplification Reagent - EnVision FLEX+ Mouse (LINKER)	AutostainerLink 48, DAKO	CTLA-4 protein expressed on some tumor and immune cells
CK5/6, Mo	D5/16 B4, Isotype: IgG1, kappa	Dako, Agilent, Glostrup, Denmark	RTU	ultraVIEW Universal DAB Detection Kit	BenchMark, Ventana	Basal cell cytokeratins 5 and 6
CK17, Mo	E3, Isotype: IgG2b, kappa	Dako, Agilent, Glostrup, Denmark	RTU	ultraVIEW Universal DAB Detection Kit	BenchMark, Ventana	Basal cell cytokeratin 17

The EnVision™ FLEX, High pH, (Link), DAKO visualization system was used for detection of most antibodies. It is intended for the IHC staining method. It has a high sensitivity for visualizing the primary antibodies bound to the relevant antigens. A double link system (Link) detects primary mouse and rabbit antibodies and the reaction is visualized by 3,3'-diaminobenzidine (DAB) + chromogen. Kit includes Peroxidase-Blocking Reagent (EnVision™ FLEX Peroxidase-Blocking Reagent), EnVision™ FLEX /HRP, EnVision™ FLEX DAB+ Chromogen, EnVision™ FLEX Substrate Buffer, High pH Target Antigen Discovery Solution (50x Tris Buffer , pH 9) EnVision™ FLEX Target Retrieval Solution, High pH (50x) and EnVision™ FLEX Wash Buffer (20x).

EnVision FLEX+ Mouse (LINKER) is an additional reagent used in combination with the other components of the imaging system (in detection of PD-L1 анд CTLA-4), allowing amplification of the primary mouse antibody signal and the reaction visualized by DAB chromogen.

The UltraVIEW Universal DAB Detection Kit imaging system (used for the detection of the basal cell cytokeratins CK17 and CK5/6) is also designed for the IHC staining method. It is a complex of biotin-free reagents with high sensitivity for visualization of primary antibodies bound to the respective antigens. The system detects primary IgG and IgM mouse and rabbit antibodies using a cocktail of secondary antibodies labeled with HRP (Horseradish Peroxidase) enzymes. This complex is visualized with DAB hydrogen peroxide and chromogen. The HRP component and biotin-free reaction flow improve the sensitivity and specificity of the IXX assay by improving reaction kinetics and reducing the possibility of background staining that occurs with exposure to biotin. The kit includes 5 reagents: DAB Inhibitor, HRP Multimer, DAB Chromogen, DAB H2O2, Copper.

Tissue sections with a thickness of 2-4 μm were prepared from FFPE tissue materials and placed on adhesive slides. Each of the 100 patients studied was tested with each of the nine primary antibodies and imaging system, using the respective AutostainerLink 48, DAKO or BenchMark, Ventana automated system.

All work procedures (including deparaffinization and rehydration of tissue sections; heat-induced antigen retrieval; incubation with primary antibody and with detection system; visualization of the complex, etc.) when performing the IHC analysis were fulfilled according to the protocols for the respective antibodies of the manufacturing company.

In each staining run, external control tissues were used to establish the functionality of the staining reagents, to assess the quality of the staining reaction, to determine the expression pattern of the antibodies used, and to optimize the IHC work procedures before applying them to the studied cases - **Table 3**.

Table 3. *Control tissues used and IHC expression pattern established for respective antibodies*

Primary antibody	Control tissue	Cellular localization	Cell positivity
CD3	tonsil	membrane and/or cytoplasmic	Moderate to strong expression in T cells of the interfollicular regions and in the germinal centers; no expression in B-cells
CD4	tonsil	membrane	Moderate to strong expression in T-helper cells of the interfollicular regions and weak to moderate in germinal center macrophages
CD8	tonsil	membrane and cytoplasmic	Moderate to strong expression in T cells from interfollicular regions
CD20	tonsil	membrane	Moderate to strong expression in mantle zone B cells and germinal centers; lack of expression in squamous epithelial cells
FoxP3	tonsil	nuclear	Moderate expression in T-regulatory cells of the interfollicular regions and in the germinal centers
PD-L1	tonsil	membrane	Strong expression in squamous epithelium from crypts; weak to moderate expression in germinal center macrophages; expression is absent in covering squamous epithelium, endothelial cells and fibroblasts
CTLA4	appendix	cytoplasmic	Glandular epithelium and lymphoid cells; lymph node - cytoplasmic and/or membrane staining in germinal center lymphoid cells
CK5/6	tonsil	cytoplasmic	Moderate and strong expression in squamous epithelial cells
CK17	skin	membrane and/or cytoplasmic	Moderate to strong expression in myoepithelial cells of sweat glands, lack of expression in squamous and glandular epithelial cells

Reporting of positive IHC-stained cells was performed independently by two pathologists who were not aware of the clinicopathological data of the cases studied.

2.3.1. Microscopic assessment of CD3, CD4, CD8, CD20 and FoxP3 expression in immune cells

Immunophenotyped lymphocytes (separately intratumoral and stromal) were counted (computer assisted) and semiquantitatively graded. The average value of the results of the examined cases with BC was taken as the borderline.

In the microscopic assessment of TILs subtypes CD3, CD4, CD8 and CD20 positive lymphocytes were counted in the tumor and stroma of five randomly selected fields, at x40 magnification (HPF). Results with an average number of up to 25 IHC-positive cells were considered low, and 25 and more positive cells - as high value of the respective TIL subtype. FoxP3-positive lymphocytes were semiquantitated in at least 10 fields of the tumor area, at high magnification. The presence of a mean number of <15 and ≥ 15 FoxP3 IHC-positive cells was counted as low and high FoxP3 expression, respectively.

In the absence of IHC positive cells, a negative result was accepted.

2.3.2. Microscopic assessment of PD-L1 and CTLA-4 IHC expression in tumor and immune cells

IHC expression of tumor and immune cells in the molecular subtypes of BC was studied, determining: localization of cellular expression (cytoplasmic/membrane/nuclear); degree of intensity - 1+ (weak), 2+ (moderate), 3+ (strong) and percentage of positive for PD-L1 and CTLA-4 tumor cells; location (intratumoral and stromal) and degree of infiltration of the primary tumor by PD-L1- and CTLA-4-positive immune cells.

Tumor cell expression was assessed on the whole tissue section by determining the percentage of viable tumor cells (the " tumor proportion" - TP) showing varying intensity, and when reporting positivity they are included partial or complete membrane staining for PD-L1 and cytoplasmic staining for CTLA-4. Results were interpreted as: negative with no PD-L1 expression (TP <1%), positive with low (TP 1-49%) or high (TP ≥ 50%) PD-L1 expression; negative with absence of CTLA4 expression or presence of only intensity 1+ tumor cells; weakly positive with CTLA4 expression of intensity 2+ and/or 3+ in 1-49% tumor cells; strongly positive with CTLA4 expression of intensity 2+ and/or 3+ in >50% tumor cells.

Tumor-associated immune cells showing partial or complete membranous PD-L1 staining of intensity 1+, 2+ and/or 3+, moderate and strong membranous and/or cytoplasmic lymphocyte IHC expression for CTLA-4 were counted separately in the tumor and the stroma of the entire tissue section. The results are assigned to the following categories: 0 (negative, with the absence of positive immune cells); low and high concentration of PD-L1 and CTLA-4 positive immune cells, after determining what part (area/percentage) of the entire tumor area is occupied by positive intratumoral and stromal immune cells. The mean value of the results of the examined cases with BC (1% for stromal and intratumoral PD-L1+ immune cells; 1% for intratumoral and 2% for stromal CTLA4+ immune cells) was taken as the cutoff.

2.3.3. Microscopic assessment of CK5/6 and CK17 expression in tumor cells

The IHC expression in tumor cells in the molecular subtypes of BC was studied, determining: localization of cellular expression (cytoplasmic / membrane / nuclear); degree of intensity - 1+ (weak), 2+ (moderate), 3+ (strong) and percentage of positive for CK5/6 and CK17 tumor cells.

Expression in tumor cells was assessed on the whole tissue section by determining the percentage of viable tumor cells showing different intensities. Basal subtype carcinomas were considered those in which IHC expression (cytoplasmic for CK5/6; cytoplasmic and/or membranous for CK17) was found in the tumor cells for both basal cell cytokeratins - CK5/6 and CK17 (with a cut-off value ≥60 %) or only for one, but in the presence of ≥80% positive tumor cells, with moderate or strong intensity, when evaluating the entire tissue section.

3. Statistical method

Analyzing the collected and summarized data, using appropriate parametric and non-parametric statistical tests.

3.1. Statistical analysis applied in the present study

The obtained results were entered and processed in software with the statistical package IBM SPSS Statistics 25.0 and MedCalc Version 14.8.1. $p<0.05$ was accepted as a level of significance at which the null hypothesis is rejected.

The following methods are applied:

1. Descriptive analysis – the frequency distribution of the considered signs, broken down by research groups, is presented in a table.
2. Analysis of Variance – to assess the characteristics of central tendency and statistical dispersion.
3. Graphical analysis – for visualization of the obtained results.
4. Comparing relative shares.
   Fisher's exact test and χ^2 test - to test hypotheses about the presence of a relationship between categorical variables.
5. Correlation analysis (Kendall's tau b) – to test hypotheses about the presence of dependence between categorical variables.
6. Kaplan-Meier method - for estimating the time until the occurrence of the studied event (Kaplan-Maier Product Limit Estimation of the Survival Function). The method is suitable for relatively small-scale studies. It follows a group of n subjects with different times of inclusion in the study and fixes the time to the occurrence of the event.

IV. RESULTS

1. Percentage distribution of the studied indicators (epidemiological, clinico-pathological and immune) in the studied cases (n=100) and in those distributed by groups (separate molecular subtypes of BC - LumA, LumB, HER2, TN, each with n=25).

1.1. Clinico-pathological and epidemiological data

The study involved 100 people with an average age of 63.90±12.17 years in the range of 35-87 years. Of them, 99 (99%) were women and 1 (1%) was a man (**Figure 2**). The age group with the largest number (27%) is 70-79 years, followed by 60-69 years with 26%, and with the smallest (3%) – 30-39 years (**Figure 3**).

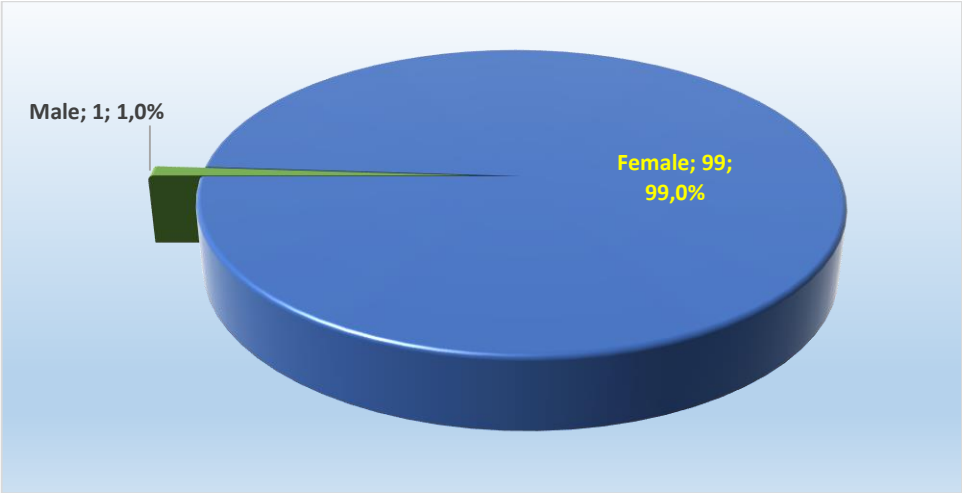


Figure 2. *Frequency distribution of study participants by sex*

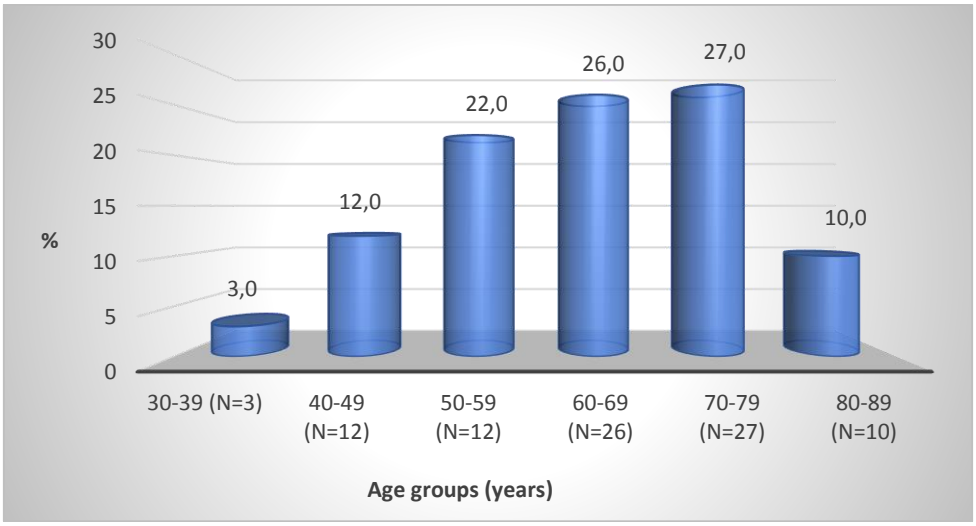


Figure 3. *Distribution of study participants by age group*

It can be seen from **Table 4** that:

- The majority of examined patients (84%) were over the age of 50 years. Those with the TN subtype had the highest relative share (96%) at this age, followed by the HER2 subtype with 84%. In patients under 50 years of age, subtype LumA was found most often (24% of cases), followed by subtype LumB (in 20%);
- Those with 5-year overall survival in the entire pollulation studied were 55%, with the highest relative proportion (92%) in the LumA subtype group, followed by those with LumB subtype at 64% and TN at 36%, and the least from HER2 subtype (28%);
- **Figure 4** shows that there is a significant difference in the cumulative survival functions of those with different degrees of differentiation:
  - the cumulative survival function of patients with HER2 positive tumors ends earlier and at the lowest level compared to those with TN, LumA and LumB carcinomas;

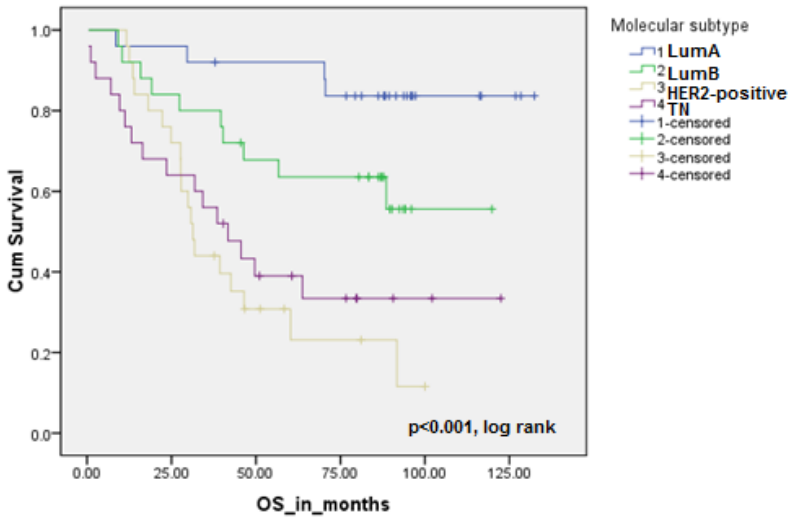


Figure 4. *Function of the overall (incl. 5-year) survival according to the indicator molecular subtype BC*

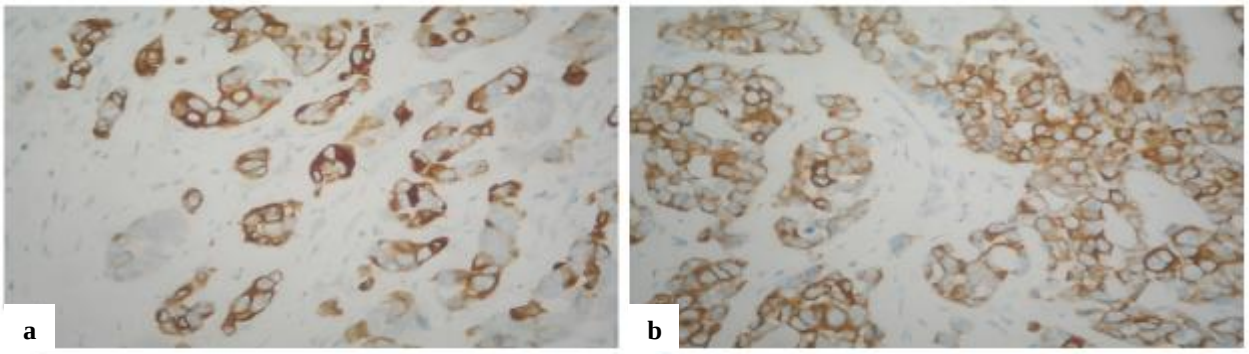
- Regarding the histological grade, the most were patients with high degree, G3 – 48% for the entire population, and it was mainly found in HER2 and TN subtypes (determined in 72% of cases in them). G2 carcinomas followed at 41% for all studied cases, with the highest percentage (68%) in the LumA subtype, followed by the LumB subtype at 44% and the lowest percentage (24%) in the HER2 subtype. The least were the patients with G1 neoplasms – 11% for the whole population, with the maximum percentage (32%) in the LumA subtype, followed by the LumB subtype with 8% and the minimum percentage (0%) in the TN subtype;
- Predominant part of the cases were in II^{-nd} clinical stage, 56% for the entire population, determined in maximum (64%) number of patients with TN and in minimum (48%) with LumA subtype. In the second place - 27% of the entire sample were in the III^{-rd} clinical stage - with a maximum percentage (32% each)

for subtypes LumB and HER2, and a minimum for subtype LumA. 16% of the examined cases with BC were in the I-th clinical stage, and the majority of them were LumA subtype (36%). Only one patient (1%) of the entire sample was assigned IV-th clinical stage, and he had HER2 subtype;

- In the majority of patients (53%), no axillary lymph node metastases were detected, with those with the LumA subtype having the highest percentage (76%), followed by the TN subtype with 64.0%. Most of the cases with metastatic lymph nodes were with LumB and HER2 subtypes (64% each);
- With regard to lymphovascular invasion - patients with no lymphovascular invasion prevailed again - 76% for the entire population, and most often they were from the LumA subtype group - 96%, followed by TN subtype with 76%. The highest percentage of those with lymphovascular invasion was observed with the HER2 subtype – 40%, followed by the LumB subtype with 28%;
- 79% of patients in the entire sample had tumors larger than 3 cm in size, with most (88% each) having LumB and HER2 subtypes. The highest percentage (36%) of tumors ≤ 3 cm were of the LumA subtype group;
- Of the histological types with the highest percentage (80%) in the entire population was non-special type (NST), followed by another morphological type with 11% [including carcinomas: mucinous (n=4); metaplastic (n=3); tubular (n=1), adenoid-cystic (n=1); with neuroendocrine (n=1) and with medullary (n=1) features] and lobular carcinoma, with 9%. NST had the largest relative share (92%) in the HER2 subtype group, lobular carcinoma - (20%) in the LumB subtype group, and another morphological type (20% each) - in LumA and TN subtypes.
- Regarding the distribution of special morphological types among the molecular subtypes of BC, 3 cases of metaplastic carcinoma, 1 patient with adenoid-cystic carcinoma and 1 with medullary features were found in TN; with the HER2 positive subtype - 1 case with mucinous carcinoma, and the remaining cases were with LumA (3 mucinous carcinomas and 1 tubular and with neuroendocrine characteristics). No specific histological variants were included in the LumB subtype group.
- Basal-like subtype – with an example visualized in **Photomicrograph 1**, was determined in (18%) of the studied cases with BC. The largest part (48%) of them was found in the group of TN subtype, followed by HER2 with 12%, LumB with 8%, and the smallest (4%) was with LumA subtype.

Table 4. *Percentage distribution of clinicopathological data in the whole sample and in different molecular subtypes of BC*

Indicator	***Subtype*** **LumA**	***Subtype*** **LumB**	***Subtype*** **HER2**	***Subtype*** **TN**	***Whole sample***
	%	%	%	%	%
Age (years)					
≤ 50	24,0	20,0	16,0	4,0	16,0
> 50	76,0	80,0	84,0	96,0	84,0
5-year overall survival					
No	8,0	36,0	72,0	64,0	45,0
Yes	92,0	64,0	28,0	36,0	55,0
Grade					
G1	32,0	8,0	4,0	0,0	11,0
G2	68,0	44,0	24,0	28,0	41,0
G3	0,0	48,0	72,0	72,0	48,0
Stage					
I	36,0	12,0	8,0	8,0	16,0
II	48,0	56,0	56,0	64,0	56,0
III	16,0	32,0	32,0	28,0	27,0
IV	0,0	0,0	4,0	0,0	1,0
Metastatic lymph nodes					
No	76,0	36,0	36,0	64,0	53,0
Yes	24,0	64,0	64,0	36,0	47,0
Lymphovascular invasion					
No	96,0	72,0	60,0	76,0	76,0
Yes	4,0	28,0	40,0	24,0	24,0
Tumor size					
≤ 3 cm	36,0	12,0	12,0	24,0	21,0
> 3 cm	64,0	88,0	88,0	76,0	79,0
Surgical specimen					
Excisional biopsy	28,0	12,0	24,0	24,0	22,0
Mastectomy	72,0	88,0	76,0	76,0	78,0
Histological type					
NST	68,0	80,0	92,0	80,0	80,0
Lobular cancer	12,0	20,0	4,0	0,0	9,0
Other morphological subtype	20,0	0,0	4,0	20,0	11,0
Basal-like					
Non-basal	96,0	92,0	88,0	52,0	82,0
Basal	4,0	8,0	12,0	48,0	18,0



Photomicrograph 1: *IHC staining with CK 17 (a) and CK 5/6 (b) in basal-like BC, x400*

1.2. Antitumor immune response data

From **Table 5**, with results visualized in **Photomicrographs 2-6**, it is clear that in terms of the percentage distribution of the amount and localization of TIL subtypes in the entire sample and individual molecular subtypes of BC:

- In IT CD3 T-lymphocytes with the highest percentage (74%) in the whole population was their low concentration, followed by the high with 14%, and in 12% of cases they were not detected. Among those having a low concentration with the highest percentage (80% each) were subtypes LumA and HER2. At the high concentration, the relative share of patients with the TN subtype was the largest (28%), followed by HER2 with 16%.
- In ST CD3 T-lymphocytes with the highest percentage (67%) in the entire population was the high concentration, followed by the low with 31%. Among the BC subtypes, the low concentration was found in the highest percentage (48%) of tumors with the LumA subtype. At the high concentration, three of the subtypes - LumB, HER2 and TN had the same highest percentage (72% each);
- Regarding IT CD4 T-helper lymphocytes, the most (57%) in the studied cases were the patients in whom they were absent, followed by those with a low concentration (35%). Among those having a low concentration, LumB subtype was in first place with 44%, followed by TN subtype with 40%. A high concentration was observed in 8% of the entire population, only in the HER2 subtype (32%);
- In ST CD4 T-helper lymphocytes with the highest percentage (82%) in the entire sample was the low concentration, which was observed in the largest part (84% each) of cases with LumA and LumB subtypes, followed by the remaining two subtype with 80% each. At the high concentration, three of the subtypes LumB, HER2 and TN had 8% each, and LumA - 4%;
- IT CD8 T - cytotoxic lymphocytes also in the highest percentage (70%) for the entire population had a low concentration. Among those with a low concentration, the highest relative proportion (80%) was HER2 subtype, followed by LumA with 72%. The high concentration was found in 8% of the entire sample,

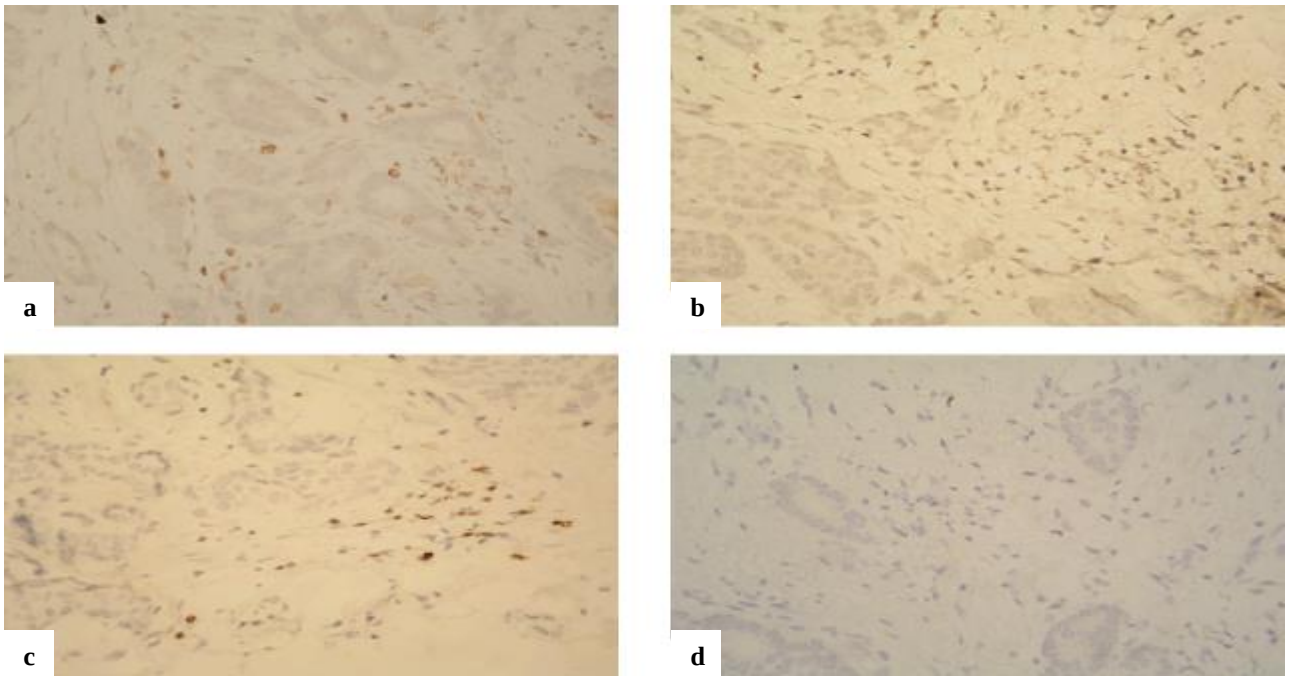
most often in the TN subtype (16%), followed by the LumB and HER2 subtypes with 8% each;

- Regarding ST CD8 cytotoxic lymphocytes - with the highest percentage (59%) in the studied patients was their low concentration, followed by the high one with 39%. Among those having a low concentration, the highest relative proportion (72%) was subtype LumA, followed by LumB with 60%. At the high concentration, the largest percentage (48%) was with the TN subtype, followed by the HER2 subtype with 44%;
- In the entire studied sample, a low concentration of IT CD20 B-lymphocytes was found most often (in 57%), and it was determined in the highest percentage (76%) in cases with the HER2 subtype, followed by the TN subtype (60%). There were no patients with a high concentration of the examined lymphocyte subtype;
- ST CD20 B-lymphocytes were also predominantly found in low and less often in high concentration in the studied cases with BC, respectively in 79% and 18%. Among those with a low concentration, the highest percentage (84%) was reported for the LumB subtype, followed by LumA and HER2 with 80% each. The highest relative share (24%) of the high concentration had the TN subtype, followed by the HER2 subtype with 20%;
- In IT FP3 T-regulatory lymphocytes with the highest percentage (68%) in the entire studied samples was the low concentration found with the highest relative proportion (80%) in patients with the HER2 subtype, followed by LumB with 76%. The high concentration (7% of the entire sample) was observed most often (12% each) in the HER2 and TN subtypes, followed by the LumB subtype with 4%;
- Again with the highest percentage (66%) in the entire studied sample was the low concentration of ST FP3 T-regulatory lymphocytes, followed by the high at 28%. Among those with a low concentration, the LumA subtype had the highest relative share (76%), followed by LumB with 72%. At the high concentration, the highest percentage (40%) was with the HER2 subtype, followed by the TN subtype with 28%.

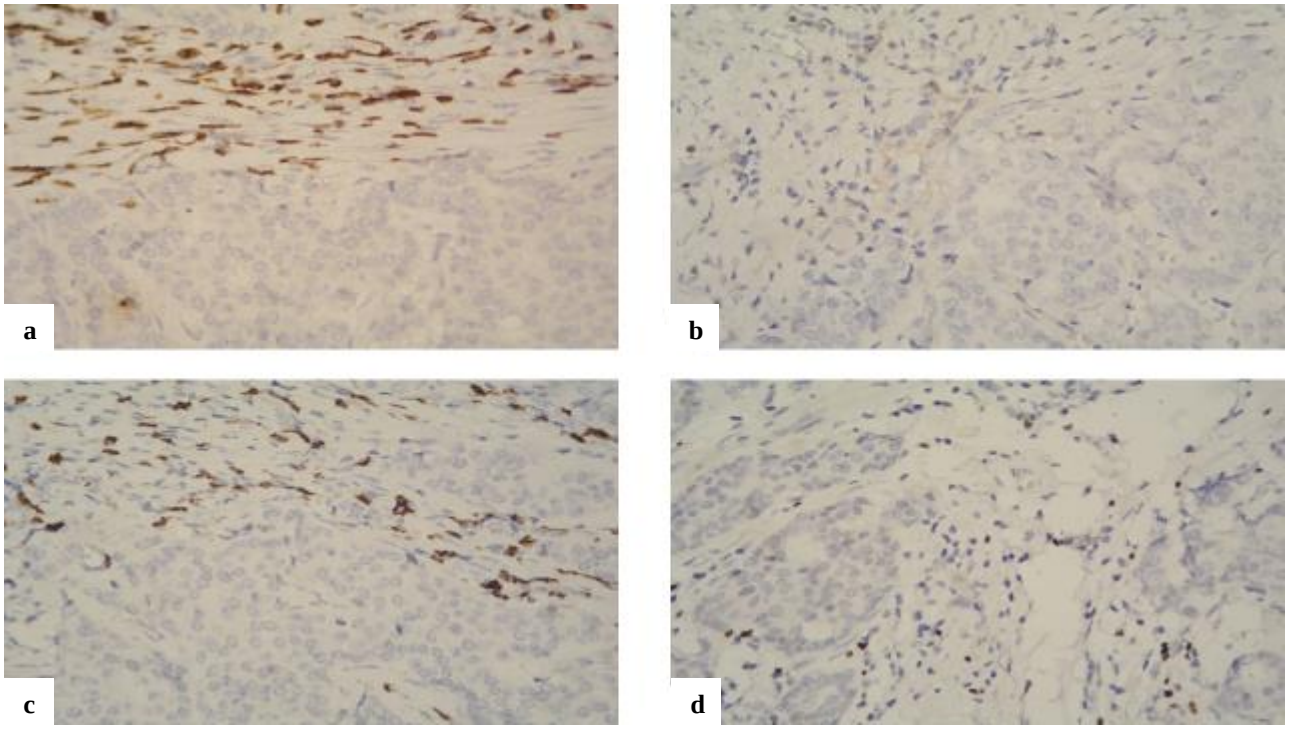
Table 5. *Percentage distribution of the amount and localization of TIL subtypes in the whole sample and in different molecular subtypes of BC*

Indicator	***Subtype* LumA**	***Subtype* LumB**	***Subtype* HER2**	***Subtype* TN**	***Whole sample***
	%	%	%	%	%
IT CD3					
Missing	20,0	12,0	4,0	12,0	12,0
Low concentration	80,0	76,0	80,0	60,0	74,0
High concentration	0,0	12,0	16,0	28,0	14,0
ST CD3					
Missing	0,0	0,0	4,0	4,0	2,0
Low concentration	48,0	28,0	24,0	24,0	31,0
High concentration	52,0	72,0	72,0	72,0	67,0

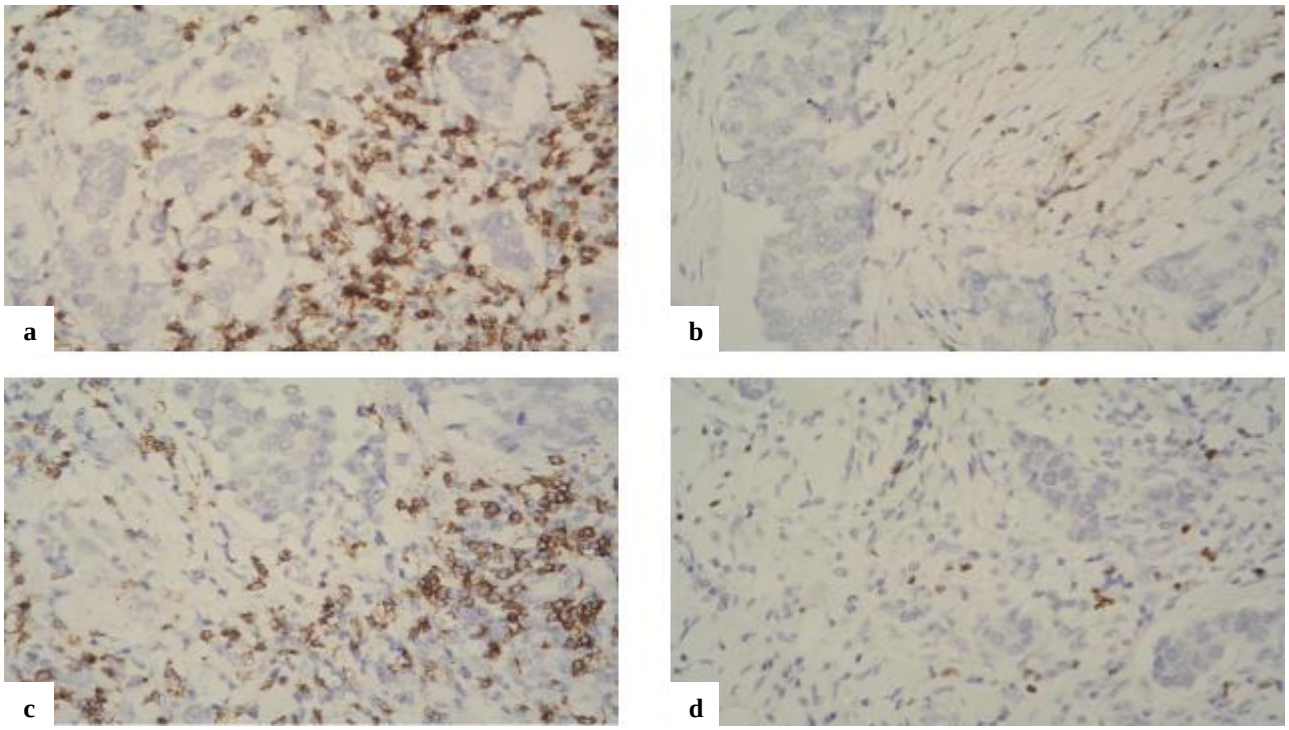
IT CD4					
Missing	72,0	56,0	40,0	60,0	57,0
Low concentration	28,0	44,0	28,0	40,0	35,0
High concentration	0,0	0,0	32,0	0,0	8,0
ST CD4					
Missing	12,0	8,0	12,0	12,0	11,0
Low concentration	84,0	84,0	80,0	80,0	82,0
High concentration	4,0	8,0	8,0	8,0	7,0
IT CD8					
Missing	28,0	28,0	12,0	20,0	22,0
Low concentration	72,0	64,0	80,0	64,0	70,0
High concentration	0,0	8,0	8,0	16,0	8,0
ST CD8					
Missing	0,0	4,0	0,0	4,0	2,0
Low concentration	72,0	60,0	56,0	48,0	59,0
High concentration	28,0	36,0	44,0	48,0	39,0
IT CD20					
Missing	56,0	52,0	24,0	40,0	43,0
Low concentration	44,0	48,0	76,0	60,0	57,0
High concentration	0,0	0,0	0,0	0,0	0,0
ST CD20					
Missing	4,0	4,0	0,0	4,0	3,0
Low concentration	80,0	84,0	80,0	72,0	79,0
High concentration	16,0	12,0	20,0	24,0	18,0
IT FP3					
Missing	44,0	20,0	8,0	28,0	25,0
Low concentration	56,0	76,0	80,0	60,0	68,0
High concentration	0,0	4,0	12,0	12,0	7,0
ST FP3					
Missing	4,0	4,0	4,0	12,0	6,0
Low concentration	76,0	72,0	56,0	60,0	66,0
High concentration	20,0	24,0	40,0	28,0	28,0



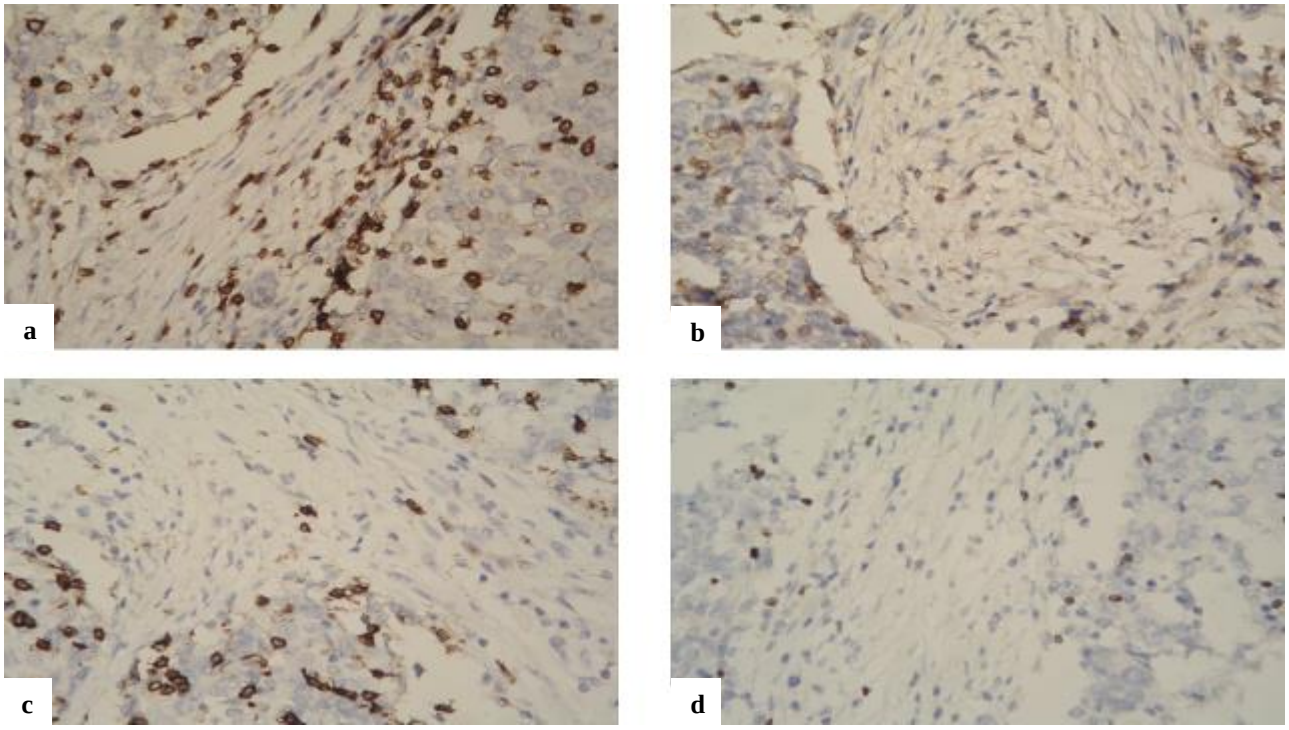
Photomicrograph 2. *Microscopic assessment of the IHC-stained T lymphocyte subtypes (a- CD3; b - CD4; c – CD8; d- FoxP3) in LumA BC, x400*



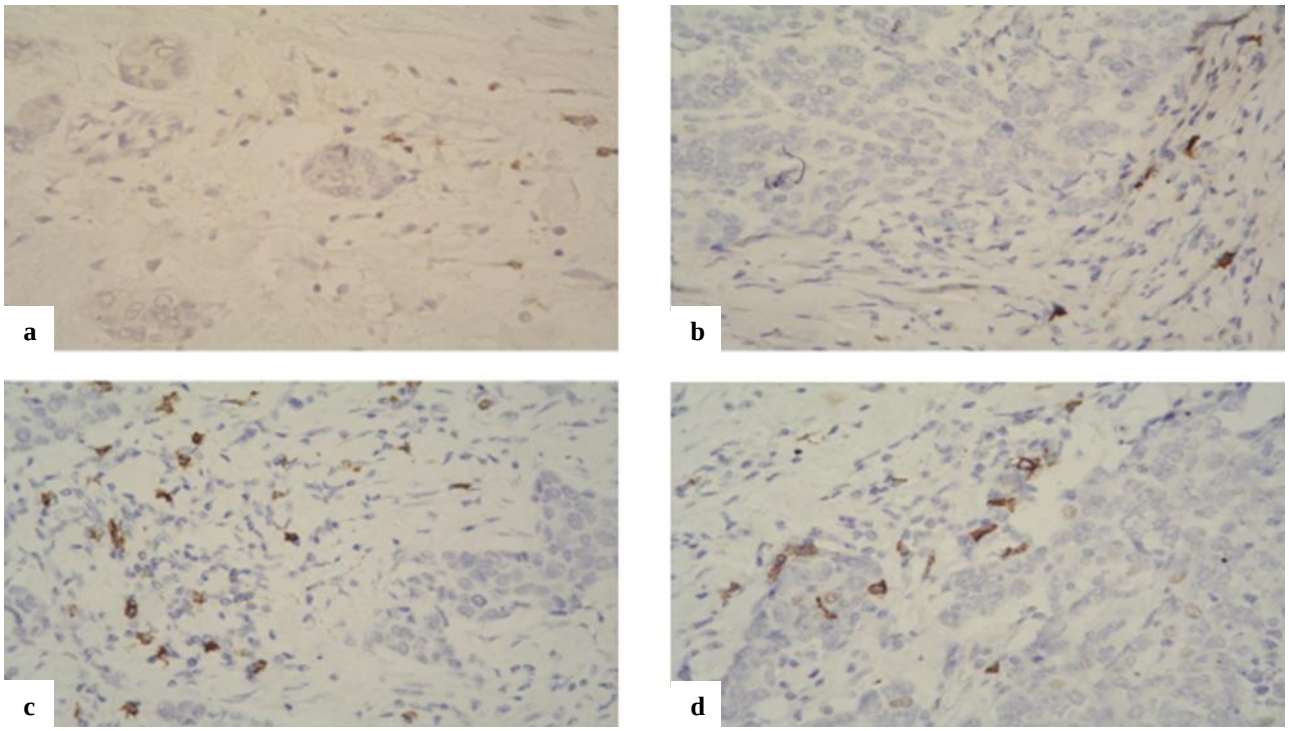
Photomicrograph 3. *Microscopic assessment of the IHC-stained T lymphocyte subtypes (a- CD3; b - CD4; c – CD8; d- FoxP3) in LumB BC, x400*



Photomicrograph 4. *Microscopic assessment of the IHC-stained T lymphocyte subtypes (a- CD3; b - CD4; c – CD8; d- FoxP3) in HER2 BC, x400*



Photomicrograph 5. *Microscopic assessment of the IHC-stained T lymphocyte subtypes (a- CD3; b - CD4; c – CD8; d- FoxP3) in TN BC, x400*



Photomicrograph 6. *Microscopic assessment of the IHC-stained B lymphocyte in different subtypes of BC (a- LumA; b- LumB; c – HER2 positive; d- TN), x400*

The results of **Table 6**, visualized in **Photomicrographs 7-9**, show that:

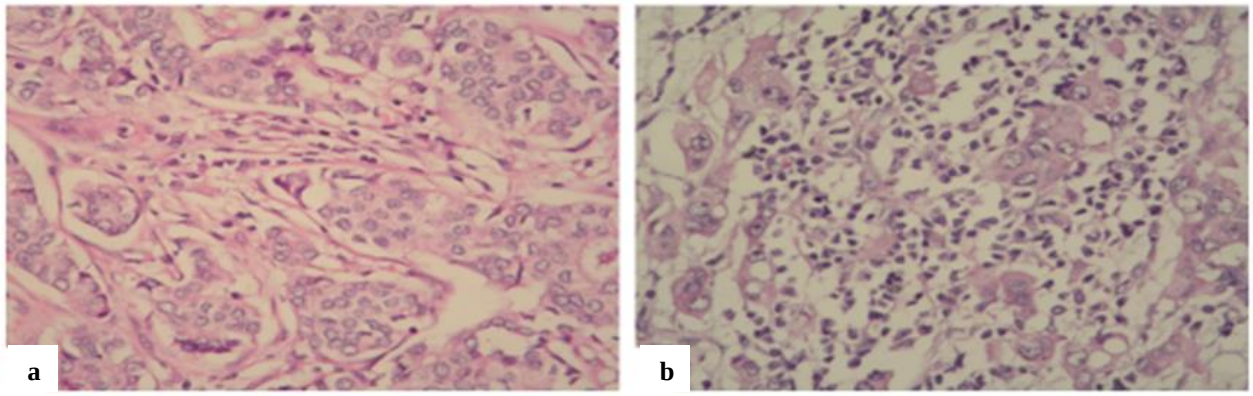
- The majority of tumors examined (89%) had a TILs ≤ 50%. With the highest relative share (100%) of them were subtype LumA, followed by subtype LumB with 96%. The highest percentage (24%) of tumors with TILs > 50% was observed with the HER2 subtype, followed by those with the TN subtype with 16%;
- In terms of IHC positivity for PD-L1 in tumor cells (% PD-L1 t.c.), most (93%) cases in the entire sample were found to be "lacking expression (negative result)", followed by those with positive result with low expression (6%). The TN subtype group had the maximum relative proportion (20%) of positive cases with low expression and included the only positive case with high PD-L1 expression in the tumor cells;
- When examining PD-L1 expression in intratumoral immune cells (% PD-L1+ IT l.), it was found that the majority (97%) of tumors in the entire studied samples lacked positive immune cells. Only 3% had a low concentration, determined in 12% of the TN subtype group. A high concentration of this indicator had not been established;
- With regard to PD-L1 expression in stromal immune cells (%PD-L1+ ST l.), the most (82%) for the entire studied samples were the cases where they were absent, followed by those with low (12%) and high concentration (6%). At the low concentration, LumB had the maximum relative share (24%), followed by TN subtypes with 16% and HER2 with 8%. The high concentration of the indicator was observed only in the group with the TN subtype (24%);

- When examining CTLA4 expression in tumor cells (% CTLA4 t.c.), the most (63%) in the entire studied samples were cases from the "Negative result with missing expression" category. These were followed by those with "Positive with low expression" (31%), found in the maximum relative proportion (48%) of tumors with HER2 subtype, followed by LumA with 36%. The "Positive with high expression" category of the metric was observed only in the LumA subtype group (24%);
- When determining CTLA4 positivity in intratumoral immune cells (%CTLA4+ IT l.) most (98% of the entire studied samples) were the cases in which it is not detected. There was no high concentration of this indicator, and only two cases (8%) from the group with the TN subtype had a low concentration;
- Regarding CTLA4 expression in stromal immune cells (%CTLA4+ ST l.), the most (50%) of the entire studied samples were the tumors in which it is not detected, followed by those with a high concentration with 33% and a low concentration (17%). At the low concentration, the maximum relative share (24%) had the HER2 subtype, followed by the LumA subtype with 20%. The high concentration of the indicator had a maximum percentage (52%) in the LumA subtype, followed by the HER2 subtype with 32%;

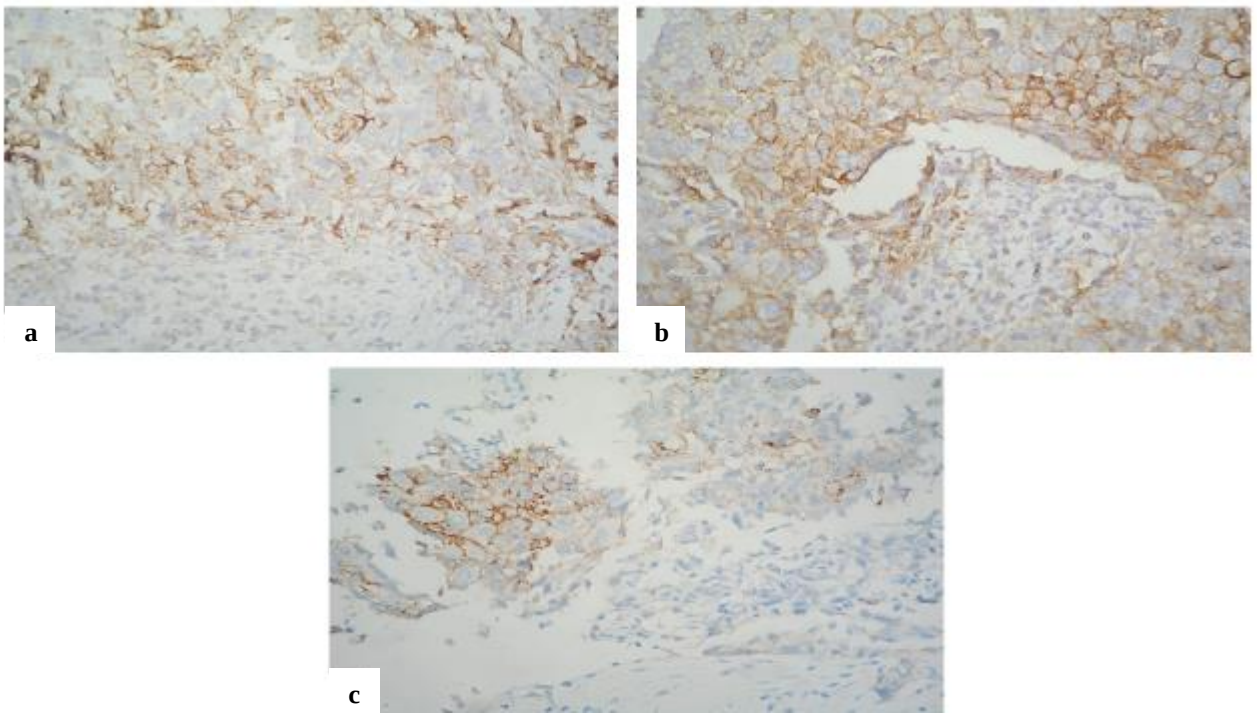
Table 6. *Percentage distribution of TIL%, PD-L1, CTLA-4 expressions in the whole sample and in different molecular subtypes of BC*

Indicator	***Subtype* LumA**	***Subtype* LumB**	***Subtype* HER2**	***Subtype* TN**	***Whole sample***
	%	%	%	%	%
TIL %					
≤ 50%	100,0	96,0	76,0	84,0	89,0
>50%	0,0	4,0	24,0	16,0	11,0
%PD-L1 t.c.					
Negative with missing expression	96,0	100,0	100,0	76,0	93,0
Positive with low expression	4,0	0,0	0,0	20,0	6,0
Positive with high expression	0,0	0,0	0,0	4,0	1,0
%PD-L1+ IT l.					
Missing	100,0	100,0	100,0	88,0	97,0
Low concentration	0,0	0,0	0,0	12,0	3,0
High concentration	0,0	0,0	0,0	0,0	0,0
%PD-L1+ ST l.					
Липсват	100,0	76,0	92,0	60,0	82,0
Low concentration	0,0	24,0	8,0	16,0	12,0
High concentration	0,0	0,0	0,0	24,0	6,0
% CTLA4 t.c.					
Negative with missing expression	40,0	84,0	52,0	76,0	63,0
Positive with low expression	36,0	16,0	48,0	24,0	31,0
Positive with high expression	24,0	0,0	0,0	0,0	6,0

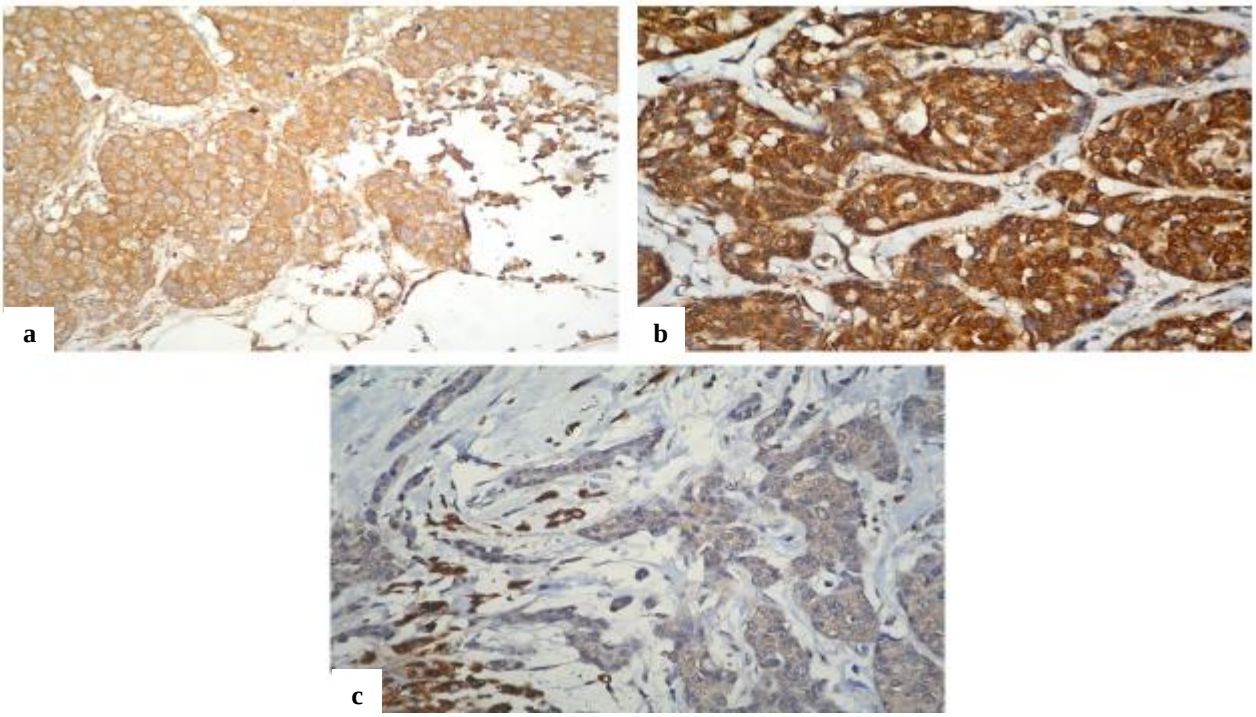
%CTLA4+ IT l.					
Missing	100,0	100,0	100,0	92,0	98,0
Low concentration	0,0	0,0	0,0	8,0	2,0
High concentration	0,0	0,0	0,0	0,0	0,0
%CTLA4+ ST l.					
Missing	28,0	64,0	44,0	64,0	50,0
Low concentration	20,0	16,0	24,0	8,0	17,0
High concentration	52,0	20,0	32,0	28,0	33,0



Photomicrograph 7. *Breast cancer with TILs ≤ 50% and TILs >50% (a, b), staining with H.E., x400*



Photomicrograph 8. *IHC assessment for PD-L1 in BC, with positive tumor and stromal immune cells (a, b, c), x400*



Photomicrograph 9.
IHC assessment for CTLA4 in BC, with different intensity in tumor cells 1+ (a), 2+ (b) and 3+ (c); positive stromal immune cells (a and c), x400

2. Comparative analysis of the studied epidemiological, clinical-pathological and immune parameters in the BC subtypes - LumA, LumB, HER2, TN (each with n=25).

2.1. Comparative analysis of the studied epidemiological and clinical-pathological indicators

The comparative analysis of the studied clinicopathological data for the individual molecular subtypes showed that significant differences were found in the indicators of 5-year overall survival, grade, clinical stage, status of lymph nodes and lymphovascular invasion (**Table 7**):

- The 5-year overall survival had a statistically significant highest percentage in patients with subtype LumA, followed by those with subtype LumB, and the lowest percentages were for the other two subtypes;
- Again, the two subtypes - LumA and LumB, had significantly higher percentages of G1 and G2 compared to the other two subtypes, while for G3 the ratio was the opposite - the higher percentages are for HER2 and TN subtypes;
- A statistically significant difference between the molecular subtypes was found only in clinical stage I – a statistically significantly higher percentage found in the LumA subtype compared to HER2 and TN (which are not statistically different from each other), but not compared to the LumB subtype, whose relative share did not differs statistically from those of the other subtypes;

- A significantly lower percentage of metastatic lymph nodes was found in the LumA subtype compared to the LumB and HER2 molecular subtypes (whose relative proportions were not statistically different from each other), but not to the TN subtype, whose relative proportions were not statistically different from those of the remaining subtypes;
- A significantly lower rate of lymphovascular invasion was found in subtype LumA compared to molecular subtypes LumB, HER2 and TN (whose relative shares did not differ statistically from each other);
- Basal-like subtype was statistically significantly found in a higher relative proportion of patients with TNBC, compared to the other three subtypes, whose percentages did not differ statistically from each other.

Table 7. *Comparative analysis of the clinicopathological data in different molecular subtypes of BC*

Indicator	***Subtype* LumA**		***Subtype* LumB**		***Subtype* HER2**		***Subtype* TN**	
	n	%	n	%	n	%	n	%
Age (years)								
≤ 50	6	24,0[a]	5	20,0[a]	4	16,0[a]	1	4,0[a]
> 50	19	76,0[a]	20	80,0[a]	21	84,0[a]	24	96,0[a]
5-year overall survival								**<0,001**
No	2	8,0[a]	9	36,0[b]	18	72,0[cd]	16	64,0[bd]
Yes	23	92,0[a]	16	64,0[b]	7	28,0[cd]	9	36,0[bd]
Grade								
G1	8	32,0[a]	2	8,0[ac]	1	4,0[bc]	0	0,0[bc]
G2	17	68,0[a]	11	44,0[ac]	6	24,0[bc]	7	28,0[bc]
G3	0	0,0[a]	12	48,0[b]	18	72,0[b]	18	72,0[b]
Stage								
I	9	36,0[a]	3	12,0[ac]	2	8,0[bc]	2	8,0[bc]
II	12	48,0[a]	14	56,0[a]	14	56,0[a]	16	64,0[a]
III	4	16,0[a]	8	32,0[a]	8	32,0[a]	7	28,0[a]
IV	0	0,0[a]	0	0,0[a]	1	4,0[a]	0	0,0[a]
Metastatic lymph nodes								**0,006**
No	19	76,0[a]	9	36,0[bc]	9	36,0[bc]	16	64,0[ac]
Yes	6	24,0[a]	16	64,0[bc]	16	64,0[bc]	9	36,0[ac]
Lymphovascular invasion								**0,027**
No	24	96,0[a]	18	72,0[b]	15	60,0[b]	19	76,0[b]
Yes	1	4,0[a]	7	28,0[b]	10	40,0[b]	6	24,0[b]
Tumor size								0,113
≤ 3 cm	9	36,0	3	12,0	3	12,0	6	24,0
> 3cm	16	64,0	22	88,0	22	88,0	19	76,0
Surgical specimen								0,552
Excisional biopsy	7	28,0	3	12,0	6	24,0	6	24,0
Mastectomy	18	72,0	22	88,0	19	76,0	19	76,0
Histological type								
NST	17	68,0[a]	20	80,0[a]	23	92,0[a]	20	80,0[a]

Lobular cancer	3	12,0[a]	5	20,0[a]	1	4,0[a]	0	0,0[a]
Other morphological subtype	5	20,0[a]	0	0,0[a]	1	4,0[a]	5	20,0[a]
Basal-like								
Non-basal	24	96,0[a]	23	92,0[a]	22	88,0[a]	13	52,0[b]
Basal	1	4,0[a]	2	8,0[a]	3	12,0[a]	12	48,0[b]

* - the same letters on the horizontal lines mean the absence of a significant difference, and the different ones - the presence of one ($p<0,05$)

2.2. Comparative analysis of the studied immune indicators

The comparative analysis of the amount and localization of TIL subtypes in the molecular subtypes of BC found that there were significant differences only in the intratumoral lymphocytes - B, T and some subtypes of T cells - helper and regulatory, respectively with indicators IT CD20, IT CD3, IT CD4 and IT FP3 (**Table 8**):

- A statistically significantly higher rate of absence of IT CD20 was found for the LumA subtype compared to the HER2 subtype, but not for the other two subtypes, whose relative proportions did not differ statistically, both among themselves and from that of the HER2 subtype; Low IT CD20 concentration was statistically significantly higher in patients with the HER2 subtype than those with the LumA subtype, but not with the other two subtypes, which were not statistically different, both by each other and by HER2 subtype. A high IT CD20 concentration was not found in any case;
- The high IT CD3 concentration had a statistically significantly higher percentage in patients with subtype TN than those with subtype LumA, but not in those of the other two subtypes, which were not statistically different from each other;
- A significantly higher percentage of missing IT CD4 was found for LumA and LumB subtypes compared to HER2 subtype, but not for TN subtype, whose relative proportion did not differ statistically from those of the other subtypes. The percentage of high IT CD4 concentration had a statistically significantly higher relative proportion in patients with the HER2 subtype compared to the other three subtypes, whose relative proportions were not statistically different from each other;
- Absence of IT FP3 had a significantly higher percentage in cases with LumA and LumB subtypes than those with HER2, but not in those with TN subtype, whose relative proportion was not statistically different from those of the other subtypes. No statistically significant differences were found for low and high IT FP3 concentration in BC subtypes.

Table 8. *Comparative analysis of the amount and localization of TIL subtypes according to different molecular subtypes of BC*

Indicator	***Subtype* LumA**		***Subtype* LumB**		***Subtype* HER2**		***Subtype* TN**	
	n	%	n	%	n	%	n	%
IT CD3								

Missing	5	20,0[a]	3	12,0[a]	1	4,0[a]	3	12,0[a]
Low concentration	20	80,0[a]	19	76,0[a]	20	80,0[a]	15	60,0[a]
High concentration	0	0,0[a]	3	12,0[ac]	4	16,0[ac]	7	28,0[bc]
ST CD3								
Missing	0	0,0[a]	0	0,0[a]	1	4,0[a]	1	4,0[a]
Low concentration	12	48,0[a]	7	28,0[a]	6	24,0[a]	6	24,0[a]
High concentration	13	52,0[a]	18	72,0[a]	18	72,0[a]	18	72,0[a]
IT CD4								
Missing	18	72,0[a]	14	56,0[a]	10	40,0[bc]	15	60,0[ac]
Low concentration	7	28,0[a]	11	44,0[a]	7	28,0[a]	10	40,0[a]
High concentration	0	0,0[a]	0	0,0[a]	8	32,0[b]	0	0,0[a]
ST CD4								
Missing	3	12,0[a]	2	8,0[a]	3	12,0[a]	3	12,0[a]
Low concentration	21	84,0[a]	21	84,0[a]	20	80,0[a]	20	80,0[a]
High concentration	1	4,0[a]	2	8,0[a]	2	8,0[a]	2	8,0[a]
IT CD8								
Missing	7	28,0[a]	7	28,0[a]	3	12,0[a]	5	20,0[a]
Low concentration	18	72,0[a]	16	64,0[a]	20	80,0[a]	16	64,0[a]
High concentration	0	0,0[a]	2	8,0[a]	2	8,0[a]	4	16,0[a]
ST CD8								
Missing	0	0,0[a]	1	4,0[a]	0	0,0[a]	1	4,0[a]
Low concentration	18	72,0[a]	15	60,0[a]	14	56,0[a]	12	48,0[a]
High concentration	7	28,0[a]	9	36,0[a]	11	44,0[a]	12	48,0[a]
IT CD20								
Missing	14	56,0[a]	13	52,0[ac]	6	24,0[bc]	10	40,0[ac]
Low concentration	11	44,0[a]	12	48,0[ac]	19	76,0[bc]	15	60,0[ac]
High concentration	0	0,0	0	0,0	0	0,0	0	0,0
ST CD20								
Missing	1	4,0[a]	1	4,0[a]	0	0,0[a]	1	4,0[a]
Low concentration	20	80,0[a]	21	84,0[a]	20	80,0[a]	18	72,0[a]
High concentration	4	16,0[a]	3	12,0[a]	5	20,0[a]	6	24,0[a]
IT FP3								
Липсват	11	44,0[a]	5	20,0[a]	2	8,0[bc]	7	28,0[ac]
Low concentration	14	56,0[a]	19	76,0[a]	20	80,0[a]	15	60,0[a]
High concentration	0	0,0[a]	1	4,0[a]	3	12,0[a]	3	12,0[a]
ST FP3								
Missing	1	4,0[a]	1	4,0[a]	1	4,0[a]	3	12,0[a]
Low concentration	19	76,0[a]	18	72,0[a]	14	56,0[a]	15	60,0[a]
High concentration	5	20,0[a]	6	24,0[a]	10	40,0[a]	7	28,0[a]

* - the same letters on the horizontal lines mean the absence of a significant difference, and the different ones - the presence of one ($p<0,05$)

The comparative analysis performed on **TIL%** indicators, **PD-L1 and CTLA-4** expressions in BC molecular subtypes showed that significant

differences were found in almost all indicators without %PD-L1+ IT l. and %CTLA4+ IT l. (**Table 9**) ;

- A statistically significantly higher relative share of TILs > 50% was found in HER2 subtype compared to LumA subtype. No statistically significant difference was found for the other two subtypes LumB and TN;
- A significantly higher rate of negative result with missing expression for PD-L1 in the tumor cells (%PD-L1 t.c.) was found for LumB and HER2 compared to TN subtype, but not for LumA, whose relative share was not differs statistically from those of the other subtypes; No statistically significant differences were found between the BC subtypes regarding the positive result with low and high expression in the tumor cells;
- The low concentration of PD-L1 positive stromal immune cells (%PD-L1+ ST l.) was significantly higher in LumB subtype than in LumA subtype, but not in the other two subtypes, whose relative proportions did not differ statistically, both among themselves and from that of the LumA subtype. The high concentration of %PD-L1+ ST l. had a statistically significantly higher percentage in subtype TN compared to the other three subtypes, whose relative shares did not differ statistically from each other;
- A significantly higher rate of negative result with missing expression for CTLA4 in tumor cells (%CTLA4 t.c.) was found in subtypes LumB and TN compared to subtype LumA, but not to subtype HER2, the relative proportion of which was significant lower than that of subtype LumB, but not statistically different from those of subtype LumA and TN. A positive result with low expression of the same indicator had a significantly higher percentage for the HER2 subtype than for the LumB subtype, but not for the other two subtypes, whose relative shares did not differ. A positive result with a high expression of %CTLA4, t.c., had a statistically significantly higher percentage in the LumA subtype compared to the other three subtypes, whose relative shares did not differ statistically from each other;
- A statistically significantly higher percentage of absent CTLA4 positive stromal lymphocytes (%CTLA4+ ST cells) was observed in subtypes LumB and TN compared to subtype LumA, but not to subtype HER2, the relative proportion of which was not statistically different from those of all remaining subtypes. The high concentration of the same indicator had a significantly higher percentage in the LumA subtype compared to that of the LumB subtype, but not to the relative proportion of those having the TN and HER2 subtypes, which were significantly higher than that of the LumB subtype group, but not to that of LumA, whose relative proportions were not statistically different from each other;

Table 9. *Comparative analysis of TILs %, PD-L1 and CTLA-4 expressions in different molecular subtypes of BC*

Показател	**Subtype LumA**		**Subtype LumB**		**Subtype HER2**		**Subtype TN**	
	n	%	n	%	n	%	n	%

TILs %								
≤ 50%	25	100,0[a]	24	96,0[ac]	19	76,0[bc]	21	84,0[ac]
>50%	0	0,0[a]	1	4,0[ac]	6	24,0[bc]	4	16,0[ac]
%PD-L1 t.c.								
Negative with missing expression	24	96,0[ac]	25	100,0[a]	25	100,0[a]	19	76,0[bc]
Positive with low expression	1	4,0[a]	0	0,0[a]	0	0,0[a]	5	20,0[a]
Positive with high expression	0	0,0[a]	0	0,0[a]	0	0,0[a]	1	4,0[a]
%PD-L1+ IT l.								
Missing	25	100,0[a]	25	100,0[a]	25	100,0[a]	22	88,0[a]
Low concentration	0	0,0[a]	0	0,0[a]	0	0,0[a]	3	12,0[a]
High concentration	0	0,0	0	0,0	0	0,0	0	0,0
%PD-L1+ ST l.								
Missing	25	100,0[a]	19	76,0[bc]	23	92,0[ac]	15	60,0[b]
Low concentration	0	0,0[a]	6	24,0[bc]	2	8,0[ac]	4	16,0[ac]
High concentration	0	0,0[a]	0	0,0[a]	0	0,0[a]	6	24,0[b]
% CTLA4 t.c.								
Negative with missing expression	10	40,0[a]	21	84,0[b]	13	52,0[ac]	19	76,0[bc]
Positive with low expression	9	36,0[ac]	4	16,0[bc]	12	48,0[a]	6	24,0[ac]
Positive with high expression	6	24,0[a]	0	0,0[b]	0	0,0[b]	0	0,0[b]
%CTLA4+ IT l.								
Missing	25	100,0[a]	25	100,0[a]	25	100,0[a]	23	92,0[a]
Low concentration	0	0,0[a]	0	0,0[a]	0	0,0[a]	2	8,0[a]
High concentration	0	0,0	0	0,0	0	0,0	0	0,0
%CTLA4+ ST l.								
Missing	7	28,0[a]	16	64,0[bc]	11	44,0[ac]	16	64,0[bc]
Low concentration	5	20,0[a]	4	16,0[a]	6	24,0[a]	2	8,0[a]
High concentration	13	52,0[a]	5	20,0[b]	8	32,0[ac]	7	28,0[ac]

* - the same letters on the horizontal lines mean the absence of a significant difference, and the different ones - the presence of one ($p<0,05$)

3. Correlation analysis of the studied indicators (epidemiological, clinico-pathological and immune) in the studied cases (n=100) and in those distributed by groups (separate molecular subtypes of BC - LumA, LumB, HER2, TN, each with n=25).

3.1. Correlations between epidemiological and clinico-pathological data (Sex, Age ≤50 / >50 years, 5 years survival, G, St, LN, LVI, tumor size, surgical specimen, Histological type).

3.1.1. Correlation analysis of **epidemiological and clinicopathological data** showed a statistically significant correlation in the entire studied samples between G and 5-year overall survival:

- **From Figure 5**, it can be seen that there was a significant difference in functions of the cumulative survival of those having different degrees of differentiation:
  - the cumulative survival function of patients with G3 tumors ends earlier and at the lowest level, compared to those with G2 and G1 carcinomas;

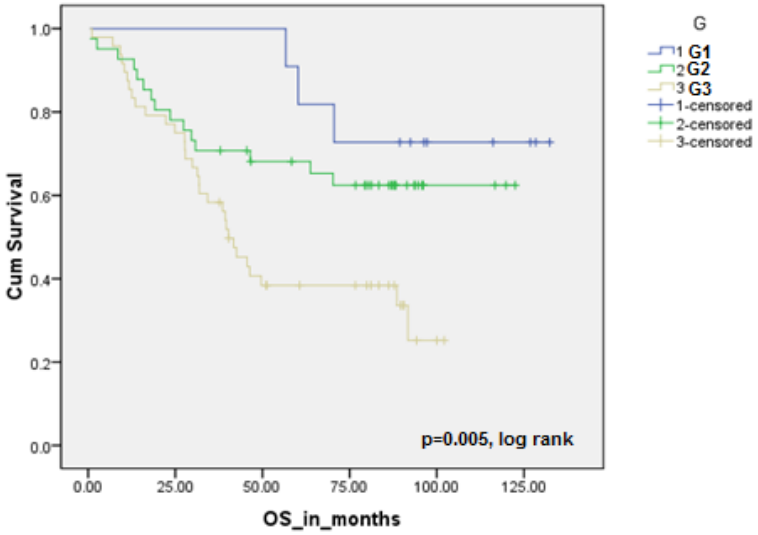


Figure 5. *Function of the overall (incl. 5-year) survival according to the indicator tumor differentiation indicator (G)*

3.1.2. The comparative analysis of the **clinico-pathological data** according to the indicator **basal-like** subtype BC showed that significant dependencies were established in the entire studied samples (**Figure 6**) and in the patients with subtype TN (**Figure 7**);

- In the entire sample, indicators that correlate with basal-like subtype of breast carcinoma were two - 5 year overall survival and histological type;

- In patients with 5-year overall survival, the relative share of the non-basal subtype of BC was statistically significantly higher, while in those without this survival, the percentage of the basal subtype was higher;
- In addition, **Figure 8** shows that there was a significant difference in the cumulative survival functions of those with basal and non-basal BC subtypes:
  - the cumulative survival function of patients with basal-type tumors ends earlier and at a lower level, compared to that of non-basal type carcinomas;
- In the category other morphological subtype of the indicator histological type, the relative share of those with basal subtype BC was significantly higher;

- Patients from the subgroup with TN subtype had a statistically significantly higher percentage of non-basal subtype BC in the presence of NST histological type, while in another morphological subtype the relative share of those with basal subtype was significantly higher.

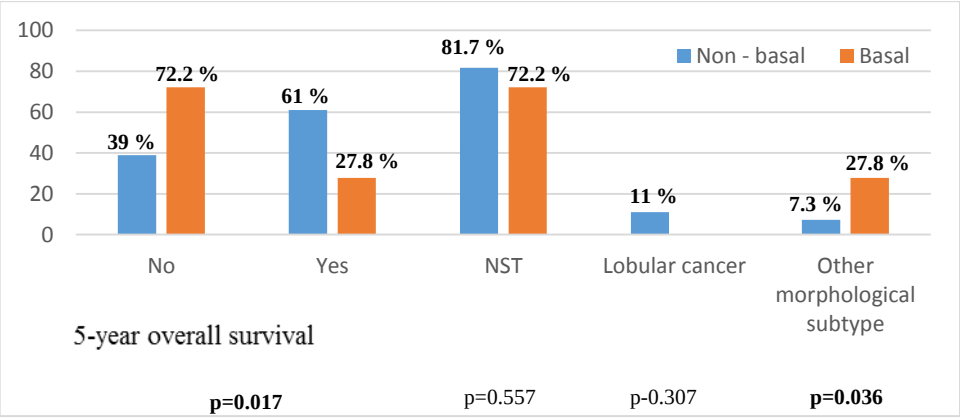


Figure 6. *Comparative analysis of clinicopathological data according to basal subtype of breast cancer (whole sample) - significant correlation with 5-year overall survival and histological type*

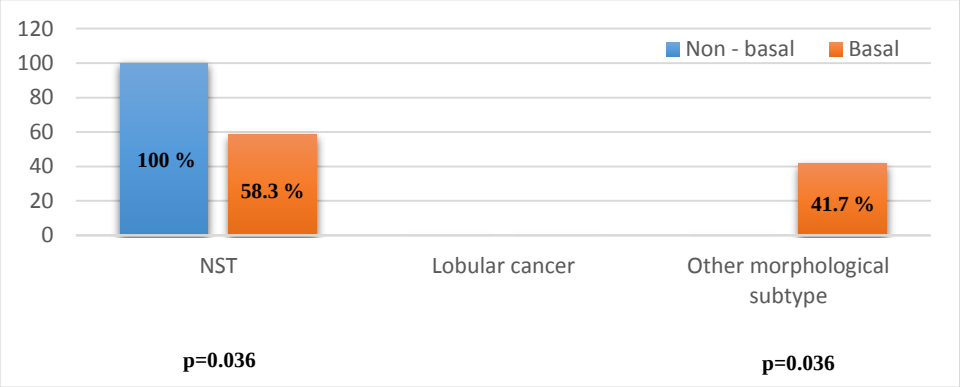


Figure 7. *Comparative analysis of clinicopathological data according to basal subtype of breast cancer (Subtype TN) - significant correlation with histological type*

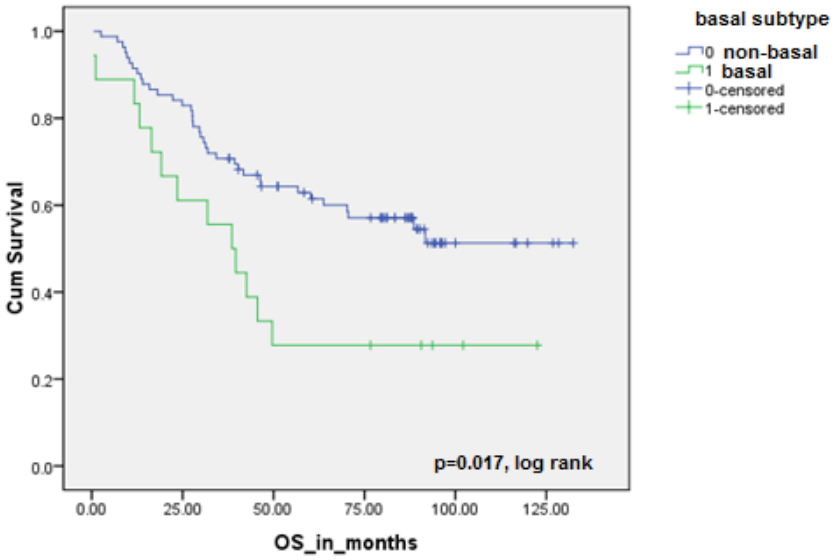


Figure 8. *Function of overall (incl. 5-year) survival according to the indicator basal subtype of BC*

3.2. Correlations between clinico-pathological data (Sex, Age ≤50 / >50, 5-years overall survival, G, St, LN, LVI, T. size, surgical specimen, histological type, basal-like subtype) **and the studied immune indicators** (TIL %, IT CD3, ST CD3, IT CD 4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3, %PD-L1 t.c., %PD -L1+ IT l., %PD-L1+ ST l., % CTLA4 t.c., %CTLA4+ IT l., %CTLA4+ ST l.)

3.2.1. Correlation analysis of **clinicopathological data** according to **TILs** values in the whole studied sample and by molecular subtypes showed that significant relationships were found only in the whole population (**Figure 9**). In subtype LumA, values of TILs > 50% are missing, and in subtype LumB this value is only one, which did not allow to draw statistically reliable conclusions in the subtypes of BC;

- In the entire sample, the indicators that correlate with TILs were two - Grade and status of lymph nodes.

- In G3 BC, a statistically significantly higher relative share of those with TILs>50% was found, while in G2, TILs ≤50% was statistically reliably found. The difference between the relative shares of TILs in G1 was statistically insignificant;
- The relative proportion of patients with TILs >50% was significantly higher in those with lymph node metastases, while that of patients with TILs ≤ 50% was greater in those without.

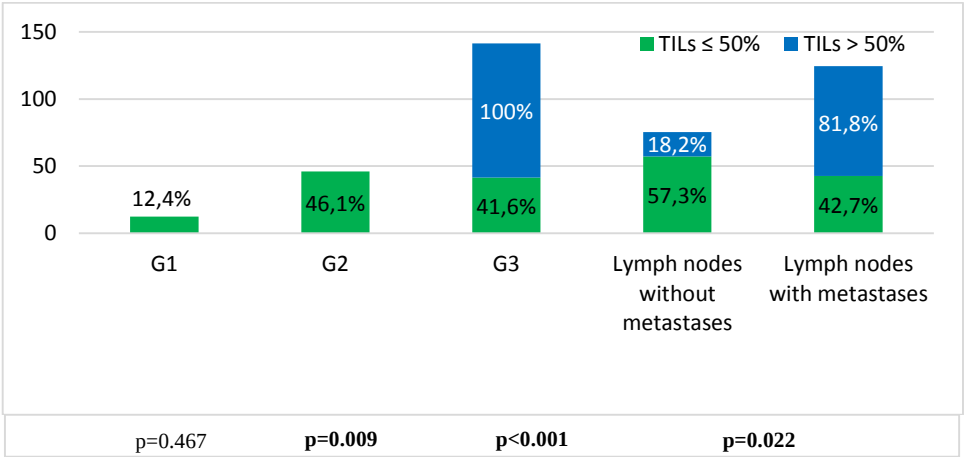


Figure 9. *Comparative analysis of clinicopathological data according to the category of %TILs (whole sample) - significant correlation with G and LN status*

- In addition, **Figure 10** shows that there was a significant difference in the cumulative survival functions of those with TILs ≤50% and TILs >50%:

- the cumulative survival function of patients with TILs >50% in their tumors ends earlier and at a lower level, compared to those with TILs ≤50%;

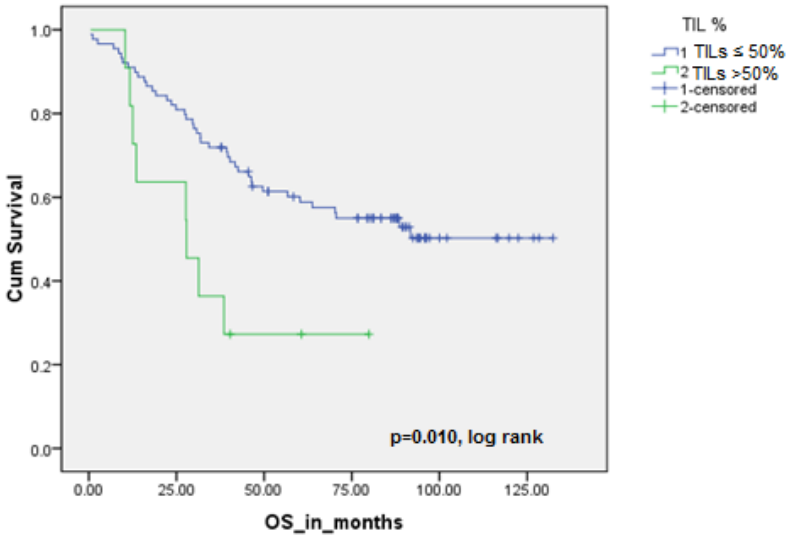


Figure 10. *Function of overall (incl. 5-year) survival according to the indicator %TILs*

3.2.2. The comparative analysis of the **clinicopathological data** according to **IT CD3** values in the whole sample and in individual molecular subtypes showed that significant dependencies were established in the whole sample and in the patients with the TN subtype (**Figures 11 and 12**);

- In the entire sample, the indicators that correlate with IT CD3 were histological degree of differentiation (Grade) and type of surgical specimen;

- In the patients with G3 BC, the relative share of those with a high IT CD3 concentration was statistically significantly greater.
- In patients with excisional biopsy, statistically significantly more often, positive IT CD3 were not detected and less often were those with a high concentration of the considered indicator, while in mastectomy it was exactly the opposite;

- Tumors of the TN subtype had a statistically significantly higher percentage of high IT CD3 concentration in G3.

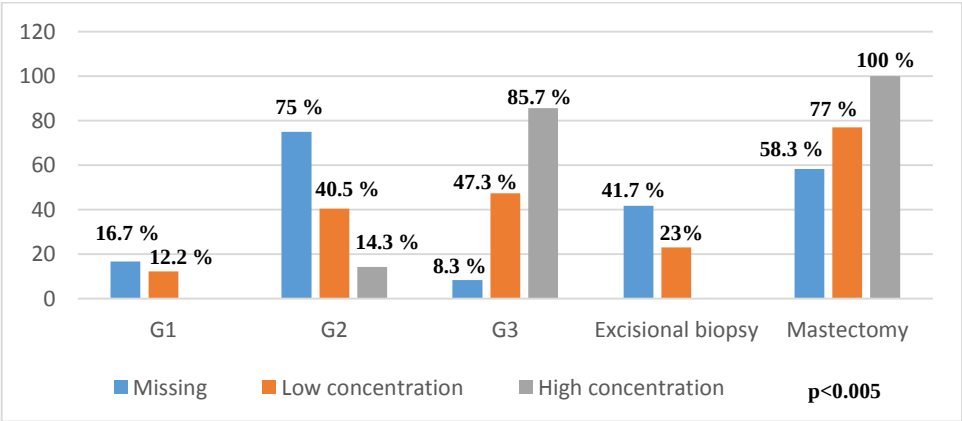


Figure 11. *Comparative analysis of clinicopathological data according to the category of IT CD3 (whole sample) - significant correlation with G and type of surgical specimen*

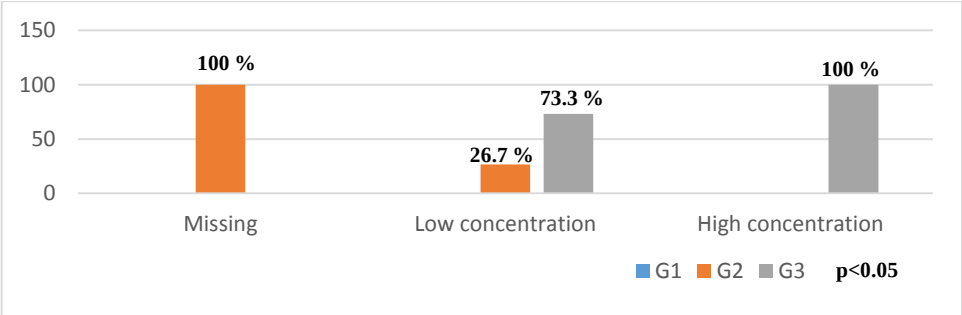


Figure 12. *Comparative analysis of the clinicopathological data according to the category of IT CD3 (Subtype TN) - significant correlation with G*

3.2.3. The correlation analysis of the **clinicopathological data** according to the **ST CD3** values in the whole studied samples and in the individual molecular subtypes showed that a significant dependence was found only in the patients with subtype LumA (**Figure 13**);

- The only indicator that correlates with the ST CD3 indicator was the type of operative material;

- In patients with excisional biopsy, it was statistically significantly higher the relative share of those with a low concentration of ST CD3, and a smaller share of those with a high concentration of the considered indicator, while the opposite was true for mastectomy. There were no patients with ST CD3 deficiency in this molecular subtype.

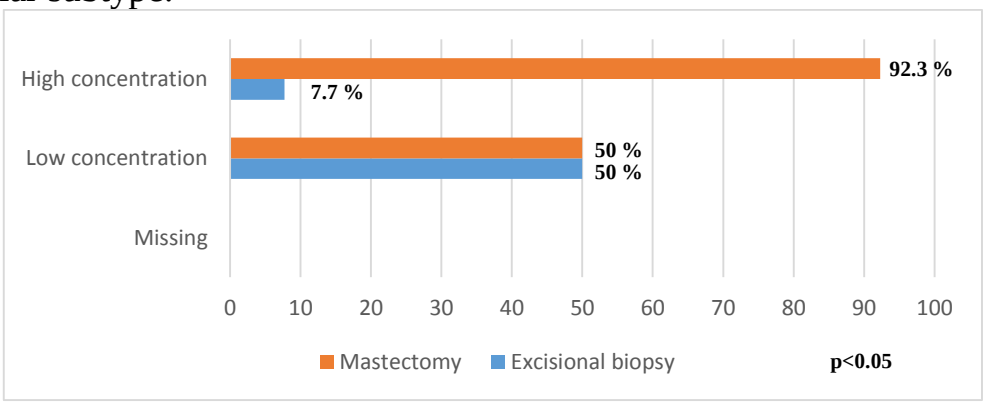


Figure 13. *Comparative analysis of the clinicopathological data according to the category of ST CD3 (Subtype LumA) - significant correlation with type of surgical specimen*

3.2.4. Correlation analysis of the **clinicopathological data** according to **IT CD4** values in the whole studied sample and in individual molecular subtypes (**Figure 14**) showed that significant dependencies were established in the whole sample, and the indicators that correlate were Grade and surgical specimen;

- In patients with G3, the relative share of those with a low concentration of IT CD4 was statistically significantly higher, and the lowest - of patients in whom this indicator is absent. The relative share of high concentration occupied an intermediate position. In G1 and G2, the difference in the frequency distribution of IT CD4 concentrations was statistically insignificant;

- In patients with excisional biopsy, it was statistically significantly higher the relative share of those without IT CD4, and least of all those with a low

concentration of the indicator under consideration, while in those with mastectomy, an opposite correlation was established; the relative share of the high concentration occupied an intermediate position.

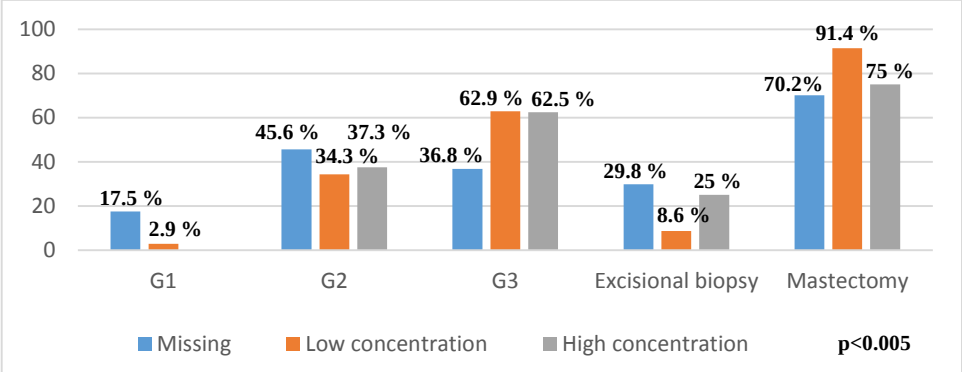


Figure 14. *Comparative analysis of clinicopathological data according to the category of IT CD4 (whole sample) - significant correlation with G and type of surgical specimen*

3.2.5. The correlation analysis of **clinico-pathological data** according to **ST CD4** values in the whole sample and for individual molecular subtypes showed that no significant relationships were established.

Correlation analysis of clinicopathological data according to IT CD8 values in the entire samples and in individual molecular subtypes (**Figures 15-17**) showed that significant dependencies were established in the entire sample and in patients with LumA and TN subtypes;

- In the entire sample and those with the TN subtype, the indicator that correlates with IT CD8 was histological type;

- In patients with another morphological subtype, significantly the highest percentage was at the high IT CD8 concentration and the smallest at the low one. The relative share of non-IT CD8 occupied an intermediate position without being statistically different from those of the other two categories;

- In the LumA subtype group, those with a tumor size ≤ 3 cm had a statistically significantly higher relative proportion of low IT CD8 concentration, while in those with a larger size, the percentage of no IT CD8. There were no patients with a high concentration of the investigated indicator in this subtype;

- In patients with TN subtype, those with NST histological type had a statistically significantly higher percentage of low IT CD8 concentration than with high, while the relative share of those without this indicator occupied an intermediate position without being statistically different from those of the other two categories.

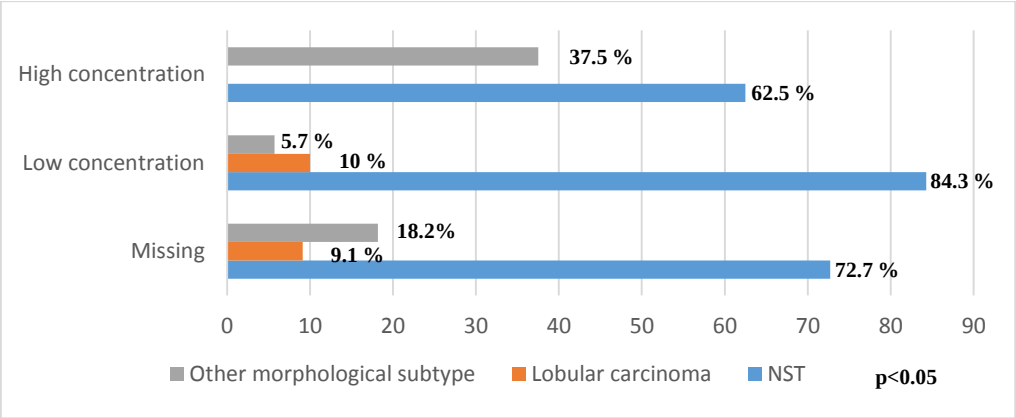


Figure 15. *Comparative analysis of clinicopathological data according to the category of IT CD8 (whole sample) - significant correlation with histological type*

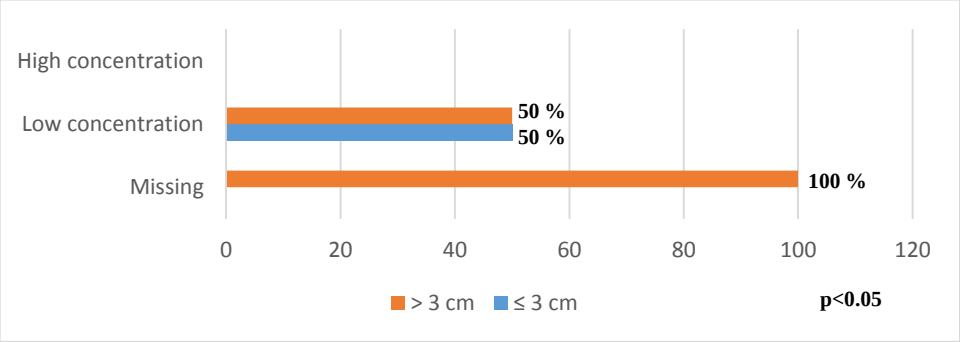


Figure 16. *Comparative analysis of clinicopathological data according to the category of IT CD8 (LumA subtype) - significant correlation with tumor size*

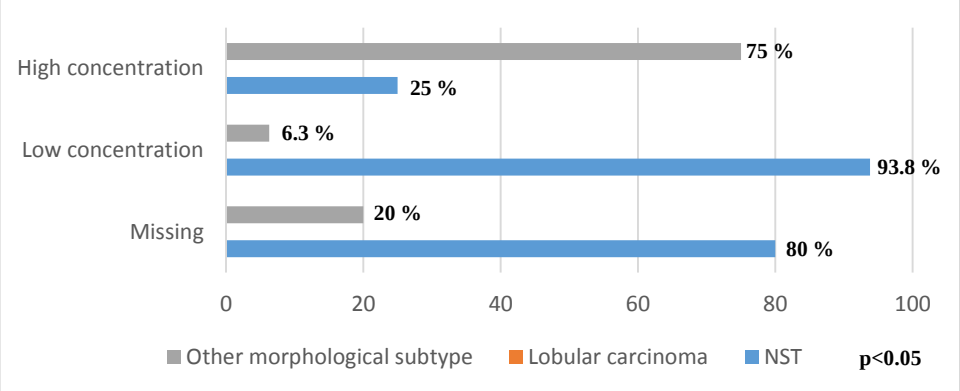


Figure 17. *Comparative analysis of clinicopathological data according to the category of CD8 IT (Subtype TN) - significant correlation with histological type*

3.2.6. The correlation analysis of the **clinicopathological data** according to the **ST CD8** values in the whole sample and in the individual molecular subtypes (**Figure 18**) showed that a significant dependence was established only in the patients of the HER2 subtype;

- The only indicator that correlates with ST CD8 was Lymphovascular invasion;

- In patients with such an invasion, there was a significantly higher relative share the high concentration of ST CD8, while in those without – the low concentration.

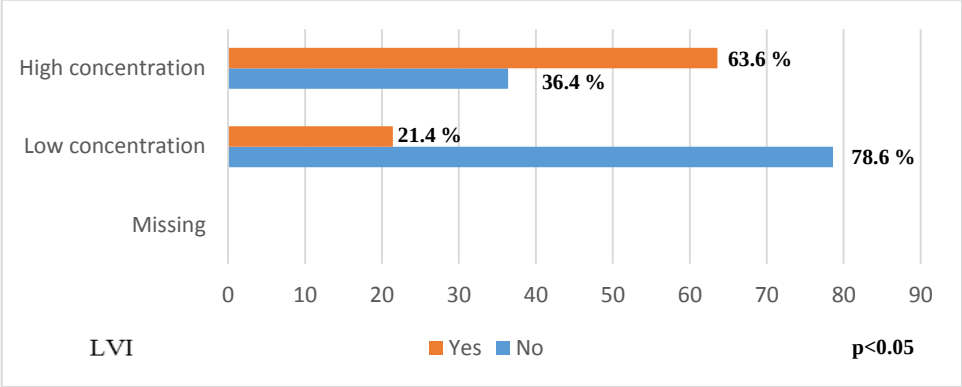


Figure 18. *Comparative analysis of the clinicopathological data according to the category of CT CD8 (Subtype HER2) - significant correlation with LVI*

3.2.7. Correlation analysis of **clinicopathological data** according to **IT CD20** values in the entire studied samples and in individual molecular subtypes (**Figure 19**) showed that significant dependencies were established in patients with the HER2 subtype;

- In the HER2 subtype group, those without 5-year overall survival had a significantly higher relative share of low IT CD20 concentration, while in those with such survival - the percentage of those without IT CD20. Patients with a high IT CD20 concentration were not identified.

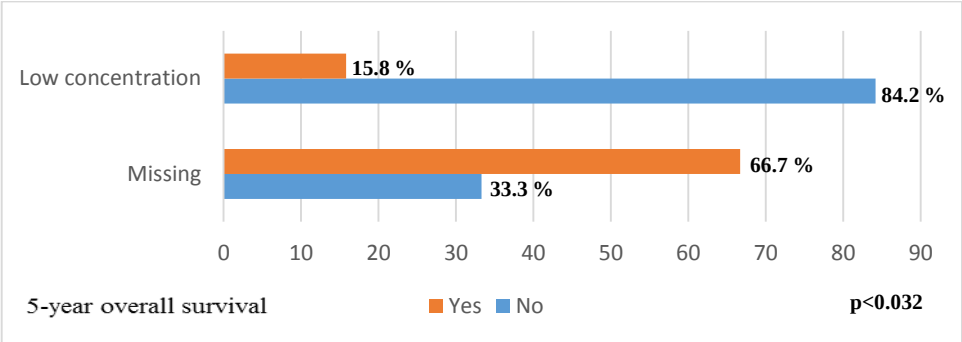


Figure 19. *Comparative analysis of clinicopathological data according to the category of IT CD20 (Subtype HER2) - significant correlation with 5-year overall survival*

3.2.8. The correlation analysis of the **clinicopathological data** according to the **ST CD20** values in the whole sample and in the individual molecular subtypes (**Figures 20 and 21**) showed that a significant dependence was established in the patients of the whole sample and those having the TN subtype;

- In the entire sample, the indicator that correlates with ST CD20 was lymphovascular invasion;

- Statistically significantly higher in those with lymphovascular invasion percent had the high concentration compared to the low one, while the opposite is true for those without LVI.

- In patients of the TN subtype, those with metastatic lymph nodes had a significantly higher percentage of high IT CD20 concentration, and those without - with a low one.

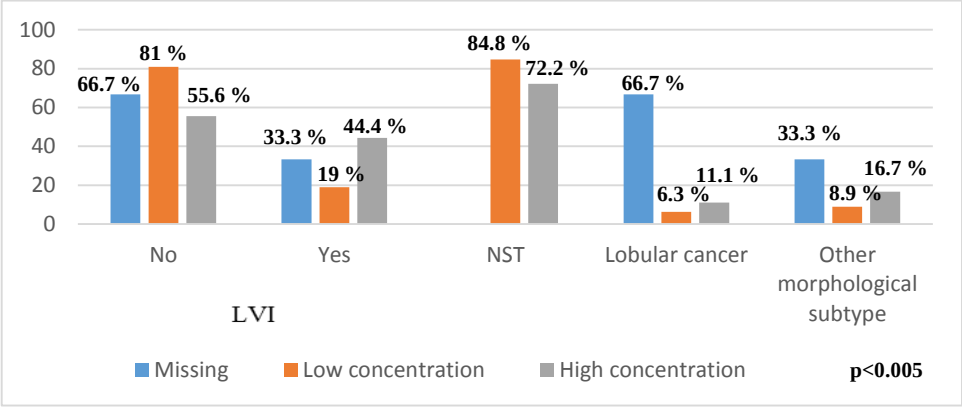


Figure 20. *Comparative analysis of the clinicopathological data according to the category of ST CD20 (whole sample) - significant correlation with LVI and histological type*

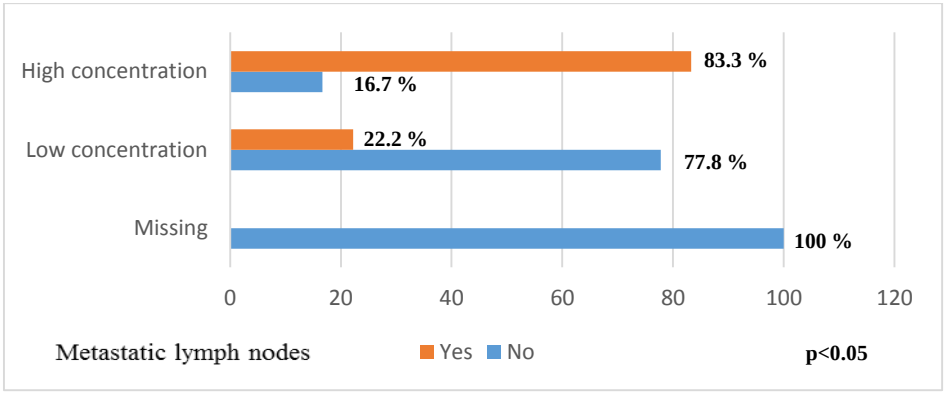


Figure 21. *Comparative analysis of the clinicopathological data according to the category of ST CD20 (Subtype TN) - significant correlation with LN status*

3.2.9. Correlation analysis of the **clinicopathological data** according to **IT FP3** values in the whole sample and in the individual molecular subtypes (**Figure 22**) showed that a significant dependence was found in the patients of the whole sample.

In the whole sample, the indicators that correlate with IT FP3 were Grade and histological type;

- In G3 carcinomas, the percentage of low and high IT FP3 concentrations (which were not statistically different from each other) was statistically significantly greater than those without this subtype of lymphocytes;
- Regarding the histological type, in the category of another morphological subtype - those without IT FP3 had a statistically significantly higher percentage compared to those with a low concentration, but not compared to those with a high concentration, whose percentage did not differ statistically from that of the other two categories. In NST and Lobular carcinoma, no significant difference was found in the frequency distribution of IT FP3;

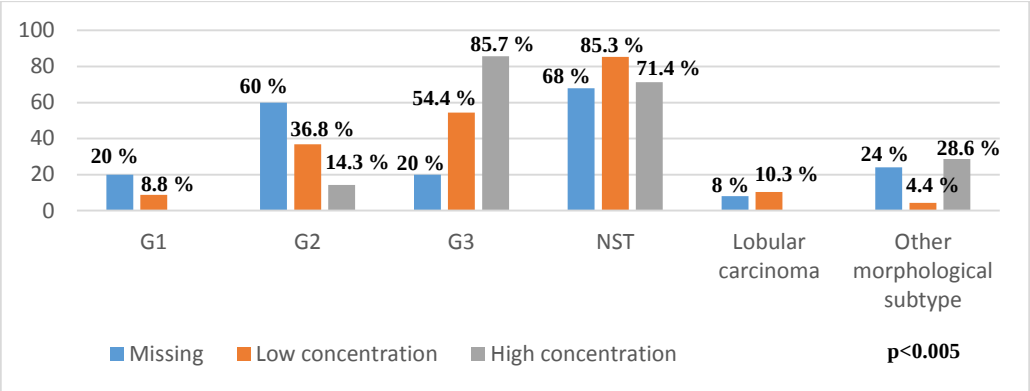


Figure 22. *Comparative analysis of clinicopathological data according to the category of IT FP3 (whole sample) - significant correlation with G and histological type*

3.2.10. Correlation analysis of the **clinicopathological data** according to **ST FP3** values in the whole sample and in the individual molecular subtypes (**Figures 23 and 24**) showed that a significant dependence was established in the patients of the whole sample and those having the LumB subtype;

- In the entire sample, the indicator that correlates with ST FP3 was Grade;

- In the G3 tumor category, the high concentration of ST FP3 had a significantly higher relative share compared to the one with low concentration, while the percentage of those without ST FP3 did not differ statistically from those of the other two categories. In G2, patients with a low concentration of ST FP3 had a statistically significantly higher relative proportion than those with a high concentration, while the percentage of those without ST FP3 was not statistically different from that of the other two categories.

- In the LumB subtype group, ST FP3 correlated with Grade and clinical stage indicators;

- Among G3 carcinomas, the percentage of those with a high concentration was significantly higher than those with a low concentration, and in this BC subtype, no cases of G3 neoplasms without FP3 cells were found;

- In patients with clinical stage II, it was statistically significantly higher the relative proportion of those having a high concentration to that of those having a low concentration. Absence of ST FP3 in this molecular subtype was observed in only one patient, therefore not included in the statistical analysis.

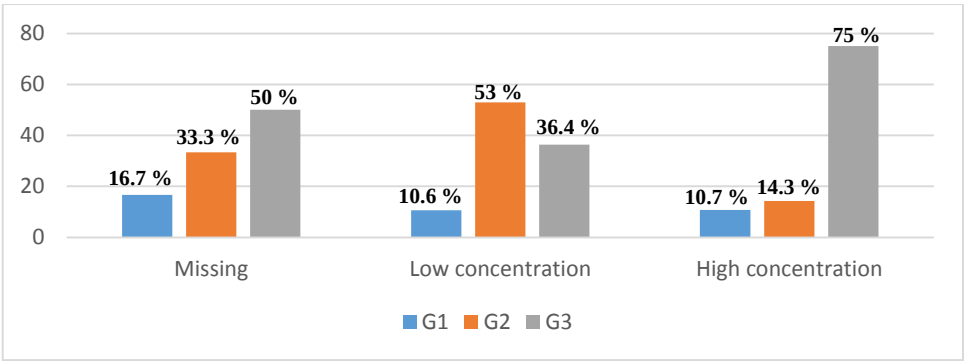


Figure 23. *Comparative analysis of the clinicopathological data according to the category of ST FP3 (whole sample) - significant correlation with G*

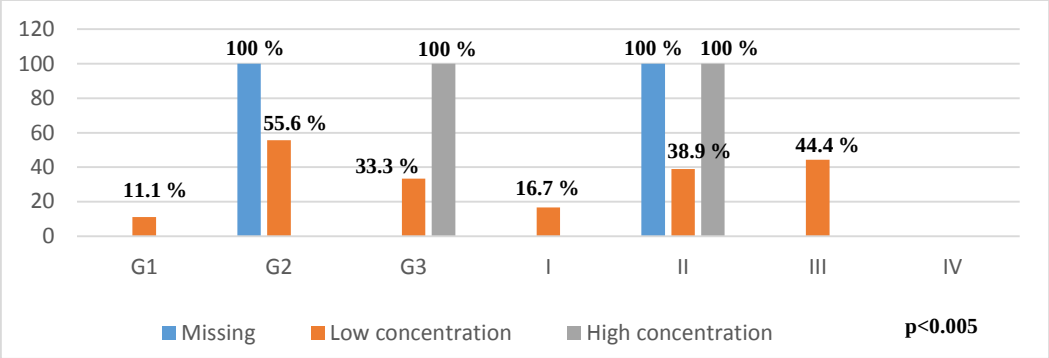


Figure 24. *Comparative analysis of the clinicopathological data according to the category of ST FP3 (LumB subtype) - significant correlation with G and stage*

3.2.11. Correlation analysis of the **clinicopathological data** according to the outcome based on the percentage of **PD-L1 positive tumor cells** in the whole sample and in the individual molecular subtypes showed, in the whole sample, only the presence of statistically significant differences in relation to the basal-like subtype carcinomas (**Table 10**):

- In case of a negative result with missing expression for PD-L1 in t.c., the relative share of the non-basal subtype of BC was significantly higher than that of the basal type; in the basal subtype, the only case with high expression of PD-L1 in the tumor cells was reported;

Table 10: *Comparative analysis of PD-L1 expression in tumor and immune cells (with intratumoral and stromal localization; low and high concentration) according to basal subtype of breast cancer (whole sample)*

Indicator	Non-basal		Basal		P
	n	%	n	%	
% PD-L1 t.c.					
Negative with missing expression	79	96,3	14	77,8	**0,023**
Positive with low expression	3	3,7	3	16,7	0,121
Positive with high expression	0	0,0	1	5,6	0,395
%PD-L1+ IT l.					0,083
Missing	81	98,8	16	88,9	
Low concentration	1	1,2	2	11,1	
%PD-L1+ ST l.					
Missing	72	87,8	10	55,6	**0,004**
Low concentration	8	9,8	4	22,2	0,287
High concentration	2	2,4	4	22,2	**0,008**

3.2.12. Correlation analysis of the **clinicopathological data** according to the score based on the percentage of **PD-L1 positive intratumoral lymphocytes** in the whole sample and in the individual molecular subtypes showed no significant associations. The percentage of patients in whom the studied indicator was missing was significantly higher. The lack of dependencies was largely

explained by the complete absence of the "High concentration" category and the presence of only 3 "Low concentration" cases in the entire sample, which were in the TN subtype.

3.2.13. Correlation analysis of the **clinicopathological data** according to the result based on the percentage of **PD-L1 positive stromal lymphocytes** in the whole sample and in the individual molecular subtypes (**Table 10 and Figure 25**) showed that the only significant relationship was with Grade, histological and basal-like type in the entire sample:

- In G3, patients with a significantly higher percentage were the patients in whom the studied indicator had a high concentration compared to those in whom it is absent, and the relative share of those with a low concentration did not differ statistically from those of the other two categories. In G1 and G2, the difference in the frequency distribution of PD-L1 positive stromal lymphocytes was statistically insignificant;
- In the "other morphological subtype" category of the histological type indicator, patients with a high concentration of PD-L1 positive stromal lymphocytes had a significantly higher relative share compared to those without them, and the relative share of those with a low concentration did not differ statistically from those of the other two categories. In the remaining two types (NST and lobular), no statistically significant difference was found in the frequency distribution of the studied indicator;
- The non-basal subtype of breast carcinoma had a statistically significantly higher percentage than the basal one in the absence of positive stromal immune cells (%PD-L1+ ST l.), while the opposite correlation was statistically reliable with their high concentration.

- The lack of dependencies in the individual molecular subtypes of BC was largely explained by the complete absence of the "High concentration" category in most of them, and in the LumA subtype also the "Low concentration" category.

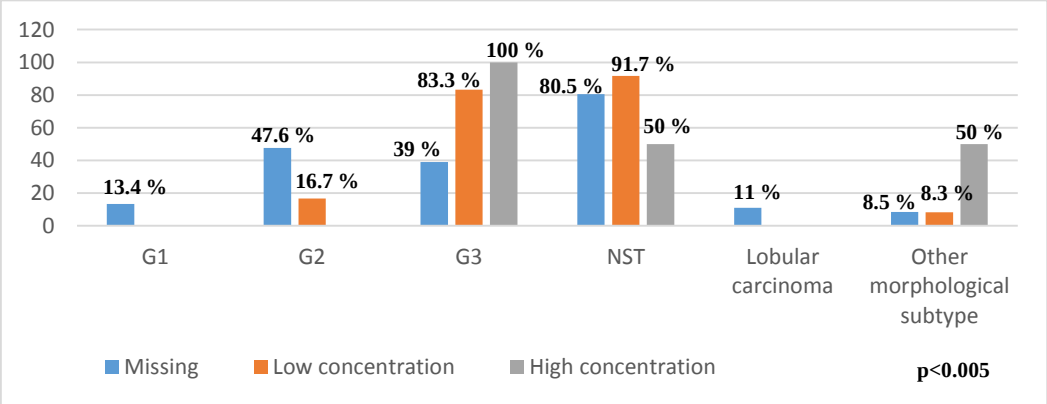


Figure 25. *Comparative analysis of clinicopathological data according to the category of concentration of PD-L1 stromal lymphocytes (whole sample) - significant correlation with G and histological type*

3.2.14. Correlation analysis of the **clinicopathological data** according to the score based on the percentage of **CTLA4 positive tumor cells** in the whole sample and in the individual molecular subtypes (**Figure 26**) showed that there was a significant relationship with Grade and histological type only in the whole sample:

- In G3, a significantly higher percentage were the patients in whom the studied indicator is negative with missing expression compared to those in whom it is positive with high expression, and the relative share of those with low expression did not differ statistically from those of the other two categories. In G1 and G2, the difference in the frequency distribution of CTLA4 positive tumor cells was statistically insignificant;
- In the "Lobular carcinoma" category of the histological type the relative share of patients with high expression of the studied indicator compared to those with low percentage was significantly higher, and the relative share of those with absent expression did not differ statistically from those of the other two categories. In the remaining two types, no statistically significant difference was found in the frequency distribution of the percentage of CTLA4 positive tumor cells.

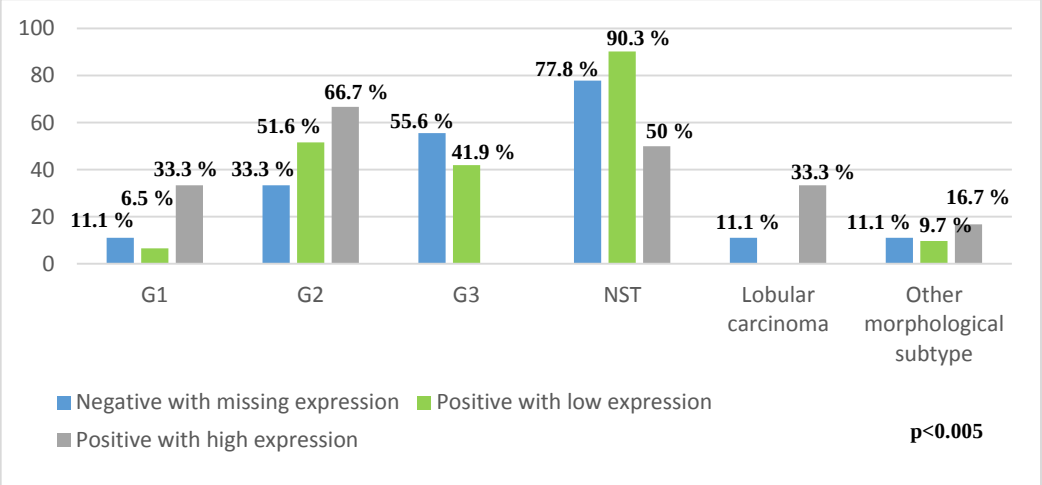


Figure 26. *Comparative analysis of clinicopathological data according to the category of CTLA4 expression in tumor cells (whole sample) - significant correlation with G and histological type*

3.2.15. Correlation analysis of **clinicopathological data** according to result based on percentage of **CTLA4 positive intratumoral lymphocytes** in the entire sample and individual molecular subtypes showed no significant relationship. The lack of dependencies was largely explained by the complete absence of the “High Concentration” and “Low Concentration” categories for almost all molecular subtypes.

3.2.16. Correlation analysis of **the clinicopathological data** according to the score based on the percentage of **CTLA4 positive stromal lymphocytes** in the entire sample and in the individual molecular subtypes (**Figures 27 and 28**)

showed that a significant relationship was found in the patients of the entire sample and those having the TN subtype;

- In the entire sample, the indicator that correlates with CTLA4 positive stromal lymphocytes was lymph node status;

- In non-metastatic LN, patients with a high concentration of the studied indicator had a statistically significantly higher relative share compared to those without it, while the percentage of those with a low concentration did not statistically differ from those of the other two categories. In the presence of metastatic lymph nodes, the inverse dependence was observed regarding the high concentration and absence of CTLA-4 positive immune cells;

- In the TN subtype group, CTLA4 positive stromal lymphocytes correlated with the lymphovascular invasion index;

- In patients with lymphovascular invasion, the percentage of those with a high concentration of the investigated indicator was significantly higher than that of those with a low concentration, and the relative share of CTLA4-positive stromal lymphocytes did not differ statistically from those of the other two categories. In patients without such an invasion, an inverse dependence was observed regarding the low and high concentration of the studied indicator.

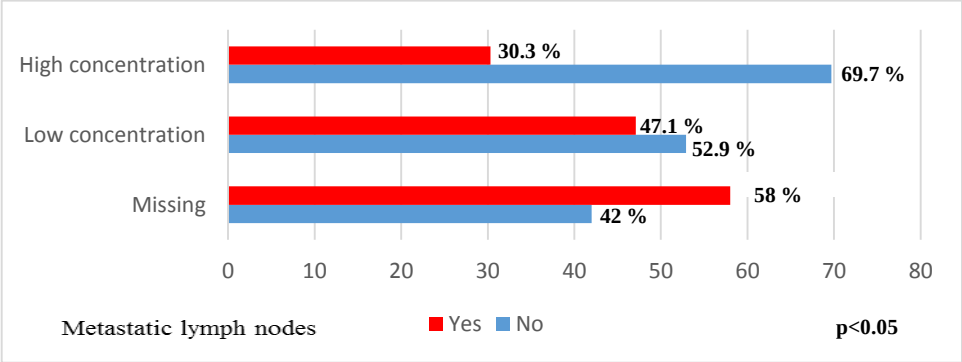


Figure 27. *Comparative analysis of clinicopathological data according to the category of concentration of CTLA4 stromal lymphocytes (whole sample) - significant correlation with LN status*

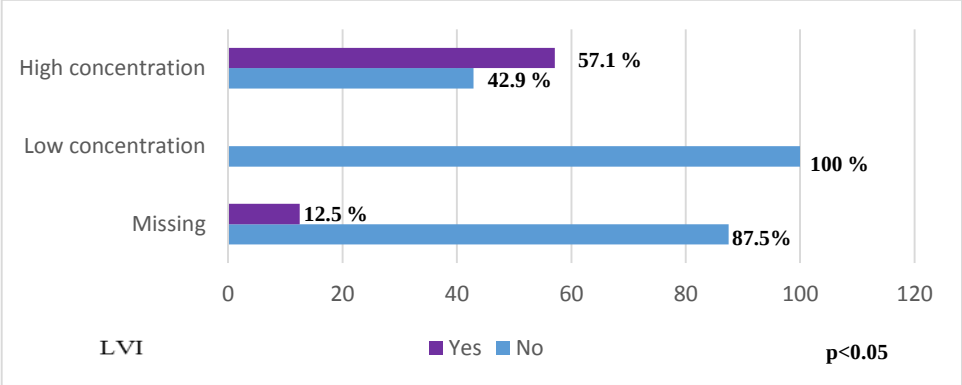


Figure 28. *Comparative analysis of the clinicopathological data according to the result based on the category of concentration of CTLA4 stromal lymphocytes (Subtype TN) - significant correlation with LVI*

4. Correlations between the amount (concentration) and localization (intratumoral - IT and stromal - ST) of the TIL subtypes (IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3) in the studied cases (n=100) and in those distributed by groups (separate molecular subtypes of BC - LumA, LumB, HER2, TN, each with n=25)

- Since the considered indicators are measured on the ordinate scale, the Kendall's tau-b coefficient was used in the correlation analysis.
- **Table 11** shows that in the entire sample:
- IT CD3 correlated weakly and directly proportionally with ST CD3, moderately and unidirectionally with IT CD4, IT CD20 and IT FP3, and strongly and directly proportionally with IT CD8;
- ST CD3 correlated moderately and unidirectionally with ST CD8, and weakly and directly proportionally with IT CD20, IT FP3 and ST FP3;
- IT CD4 correlated weakly and directly with IT CD8, and moderately and unidirectionally with IT CD20 and IT FP3;
- ST CD4 correlated weakly and directly proportionally with IT FP3, and moderately and unidirectionally with ST FP3;
- IT CD8 correlated moderately and unidirectionally with IT CD20 and IT FP3, and weakly and directly proportionally with ST FP3;
- ST CD8 correlated moderately and unidirectionally with ST FP3;
- IT CD20 correlated moderately and unidirectionally with IT FP3 and ST FP3;
- ST CD20 correlated weakly and directly proportionally with ST FP3;
- IT FP3 correlated moderately and unidirectionally with ST FP3.

Table 11. *Correlation between the amount (concentration) and localization (intratumoral and stromal) of TIL subtypes - IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3 (whole sample)*

Indicators	ST CD3	IT CD4	ST CD4	IT CD8	ST CD8	IT CD20	ST CD20	IT FP3	ST FP3
IT CD3	0,221*	0,332***	0,047	0,575***	-0,029	0,456***	0,030	0,430***	0,119
ST CD3		0,051	0,096	0,135	0,372***	0,202*	0,049	0,196*	0,297**
IT CD4			0,138	0,245**	-0,083	0,326**	0,140	0,437***	0,049
ST CD4				0,105	0,004	0,154	0,132	0,233*	0,339***
IT CD8					0,173	0,446***	0,054	0,454***	0,280**
ST CD8						0,113	0,077	0,002	0,346***
IT CD20							0,118	0,414***	0,314**
ST CD20								0,035	0,272**
IT FP3									0,339***

* - p<0,05, ** - p<0,01, *** - p<0,001

From **Table 12** it was clear that in patients with the LumA subtype:

- IT CD3 correlated strongly and directly proportionally with IT CD8;
- ST CD3 correlated moderately and unidirectionally with ST CD8;
- IT CD4 correlated strongly and directly proportionally with IT CD20, and moderately and unidirectionally with ST CD20;
- ST CD4 did not correlate with the indicators considered in the row;
- IT CD8 correlated strongly and unidirectionally with IT CD20 and IT FP3;
- ST CD8 did not correlate with the indicators considered in the row;
- IT CD20 correlated moderately and unidirectionally with IT FP3;
- ST CD20 did not correlate with the indicators considered in the row;
- IT FP3 did not correlate with the considered indicator of the row.

Table 12. *Correlation between the amount (concentration) and localization (intratumor and stromal) of TIL subtypes - IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3 (LumA subtype)*

Indicators	**ST CD3**	**IT CD4**	**ST CD4**	**IT CD8**	**ST CD8**	**IT CD20**	**ST CD20**	**IT FP3**	**ST FP3**
IT CD3	0,320	0,312	0,150	0,579**	-0,134	0,242	0,147	0,363	0,147
ST CD3		0,064	0,017	0,292	0,421*	0,206	0,071	0,277	0,323
IT CD4			0,344	0,389	-0,190	0,524*	0,446*	0,373	-0,033
ST CD4				0,325	-0,096	0,380	0,284	0,225	0,285
IT CD8					0,190	0,553**	0,183	0,524*	0,392
ST CD8						0,165	0,236	-0,165	0,359
IT CD20							0,142	0,461*	0,384
ST CD20								-0,119	0,306
IT FP3									0,295

* - p<0,05, ** - p<0,01, *** - p<0,001

The results of **Table 13** shown that in the group having the LumB subtype:

- IT CD3 correlated strongly and directly proportionally with IT CD8 and IT FP3, and moderately and unidirectionally with IT CD20;
- ST CD3 correlated moderately and unidirectionally with IT CD8 and ST FP3, and strongly and directly proportionally with ST CD8;
- IT CD4 did not correlate with the indicators considered in the table;
- ST CD4 correlated moderately and unidirectionally with CT FP3;
- IT CD8 correlated moderately and unidirectionally with ST CD8, IT CD20 and IT FP3;
- ST CD8 correlated moderately and unidirectionally with IT CD20, and strongly and directly proportionally with ST FP3;

- IT CD20 correlated moderately and unidirectionally with IT FP3 and ST FP3;
- ST CD20 did not correlate with the indicators considered in the table;
- IT FP3 did not correlate with the considered indicator of the row.

Table 13: *Correlation between the amount (concentration) and localization (intratumoral and stromal) of TIL subtypes - IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3 (LumB subtype)*

Indicators	ST CD3	IT CD4	ST CD4	IT CD8	ST CD8	IT CD20	ST CD20	IT FP3	ST FP3
IT CD3	0,353	0,320	-0,192	0,689**	0,250	0,476*	0,184	0,686**	0,126
ST CD3		0,014	0,000	0,404*	0,509*	0,243	0,344	0,359	0,419*
IT CD4			0,198	0,173	0,051	0,116	0,225	0,295	0,119
ST CD4				-0,136	0,000	0,000	0,000	0,215	0,408*
IT CD8					0,454*	0,465*	-0,119	0,452*	0,291
ST CD8						0,457*	0,060	0,174	0,621**
IT CD20							0,206	0,499*	0,418*
ST CD20								0,285	0,121
IT FP3									0,303

* - p<0,05, ** - p<0,01, *** - p<0,001

From a table **Table 14** it is clear that that in patients with HER2 subtype:

- IT CD3 correlated moderately and directly with IT CD8;
- ST CD3 did not correlate with the indicators considered in the table;
- IT CD4 correlated strongly and proportionally with IT FP3;
- ST CD4 correlated moderately and unidirectionally with ST FP3;
- ST CD8 did not correlate with the indicators considered in the table;
- IT CD20 did not correlate with the indicators considered in the table.
- ST CD20 correlated moderately and unidirectionally with ST FP3.
- IT FP3 did not correlate with the considered indicator of the row.

Table 14: *Correlation between the amount (concentration) and localization (intratumoral and stromal) of TIL subtypes - IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3 (HER2 subtype)*

Indicators	ST CD3	IT CD4	ST CD4	IT CD8	ST CD8	IT CD20	ST CD20	IT FP3	ST FP3
IT CD3	0,205	-0,089	0,229	0,419*	0,119	0,358	0,088	0,143	0,245
ST CD3		0,152	-0,051	0,000	0,372	0,302	-0,322	0,296	0,245
IT CD4			0,000	-0,196	-0,112	0,059	0,174	0,508**	-0,136
ST CD4				0,170	-0,102	0,155	0,253	0,198	0,387*
IT CD8					0,086	0,364	0,262	0,000	0,334
ST CD8						0,121	-0,242	-0,078	-0,170

IT CD20		0,047	0,255	0,329
ST CD20			0,175	0,398*
IT FP3				0,372

* - p<0,05, ** - p<0,01, *** - p<0,001

From **Table 15**, it is clear that those with the TN subtype:

- IT CD3 correlated strongly and directly proportionally with IT CD4, IT CD8 and IT CD20, and moderately and cross-directionally with ST CD8;
- ST CD3 did not correlate with the indicators considered in the table;
- IT CD4 correlated strongly and directly proportionally with IT CD8 and IT CD20, and moderately and unidirectionally with IT FP3;
- ST CD4 did not correlate with the indicators considered in the table;
- IT CD8 correlated strongly and unidirectionally with IT FP3;
- ST CD8 correlated strongly and unidirectionally with ST FP3;
- IT CD20 did not correlate with the indicators considered in the row;
- ST CD20 did not correlate with the indicators considered in the table;
- IT FP3 did not correlate with the considered indicator of the row.

Table 15: *Correlation between the amount (concentration) and localization (intratumoral and stromal) of TIL subtypes - IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3 (TN subtype)*

Indicators	**ST CD3**	**IT CD4**	**ST CD4**	**IT CD8**	**ST CD8**	**IT CD20**	**ST CD20**	**IT FP3**	**ST FP3**
IT CD3	-0,020	0,712***	0,030	0,537**	-0,389*	0,587**	-0,220	0,351	-0,047
ST CD3		-0,064	0,372	-0,211	0,161	0,014	0,091	-0,166	0,200
IT CD4			0,071	0,574**	-0,170	0,500*	-0,320	0,450*	0,075
ST CD4				0,137	0,165	0,111	0,000	0,275	0,297
IT CD8					-0,120	0,338	-0,075	0,585**	0,137
ST CD8						-0,365	0,161	-0,065	0,555**
IT CD20							0,014	0,300	0,087
ST CD20								-0,126	0,240
IT FP3									0,304

* - p<0,05, ** - p<0,01, *** - p<0,001

5. **Correlation between the expression of PD-L1 in tumor and immune cells** (with intratumoral and stromal localization; low and high concentration) - %PD-L1 t.c., %PD-L1+ IT l., %PD-L1+ ST l.) in the studied cases (n=100) and in those distributed by groups (separate molecular subtypes of BC - LumA, LumB, HER2, TN, each with n=25)

The conducted correlation analysis between the expression of PD-L1 in tumor and immune cells (with intratumoral and stromal localization) showed that (**Table 16**) in the entire sample there was a statistically reliable directly proportional correlation between the three investigated indicators - %PD-L1

t.c., %PD-L1+ IT l. and %PD-L1+ ST l., as between %PD-L1 t.c. and %PD-L1+ IT l. it was expressed in strength, and between the other two pairs – moderate;

- The only subtype in which there was also a correlation is TN, which is unidirectional and expressed in strength between the three indicators of PD-L1.

6. Correlation between the expression of PD-L1 in tumor and immune cells (with intratumoral and stromal localization; low and high concentration - %PD-L1 t.c., %PD-L1+ IT l., %PD-L1+ ST l.) **and the other studied immune indicators** (TIL%, IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT SD20, ST CD20, IT FP3, ST FP3; CTLA4 t.c., %CTLA4+ IT l., %CTLA4+ ST l.;) in the studied cases (n=100) and in those distributed by groups (separate molecular subtypes of BC - LumA, LumB, HER2, TN, each with n=25)

6.1. The correlation analysis of **PD-L1 expression in tumor and immune cells** according to **TIL subtypes** showed that statistically significant correlations in the whole sample and in the LumB and TN subtypes (**Table 17**):

- Across the **whole sample:**

- %PD-L1 t.c. correlated weakly and unidirectionally with IT CD3, IT CD20 and IT FP3, moderately and directly proportional to IT CD8, strongly and unidirectionally with %CTLA4+ IT l.;

- %PD-L1+ IT l. correlated weakly and unidirectionally with IT CD3 and IT CD8, and strongly and directly proportionally with %CTLA4+ IT l.;

- %PD-L1+ ST l. correlated moderately and directly with IT CD3, IT FP3 and %CTLA4+ IT l., weakly and unidirectionally with IT CD4, IT CD8, IT CD20 and ST FP3, and weakly and unidirectionally with % CTLA4 t.c. and %CTLA4+ ST l.;

- In the group of **LumB** subtype, only %PD-L1+ ST l. correlated directly and moderately strongly with ST CD4, directly proportionally and strongly with ST FP3, and inversely proportionally and moderately with %CTLA4+ ST l.;

- In the **TN** subtype group:

- %PD-L1 t.c. correlated moderately and unidirectionally with IT CD3, IT CD20, IT FP3 and %CTLA4+ IT l., and strongly and directly proportionally with IT CD8;

- %PD-L1+ IT l. correlated moderately and unidirectionally with IT CD8, and strongly and directly proportionally with %CTLA4+ IT l.;

- %PD-L1+ ST l. correlated moderately and directly with IT CD3, IT CD20 and %CTLA4+ IT l., moderately and unidirectionally with ST CD3, and strongly and directly with IT CD4, IT CD8 and IT FP3.

6.2. Correlation analysis of **PD-L1 expression in tumor and immune cells** (with intratumoral and stromal localization; low and high concentration) according to **%TILs** values, both in the whole studied samples and in the four molecular subtypes, found no significant differences.

Table 16: *Correlation between the expression of PD-L1 in tumor and immune cells (with intratumoral and stromal localization) - %PD-L1 t.c., %PD-L1+ IT l., %PD-L1+ ST l.*

Indicators	*Whole sample*		Subtype LumA		Subtype LumB		Subtype HER2		Subtype TN	
	%PD-L1+ IT l.	%PD-L1+ ST l.	%PD-L1+ IT l.	%PD-L1+ ST l.	%PD-L1+ IT l.	%PD-L1+ ST l.	%PD-L1+ IT l.	%PD-L1+ ST l.	%PD-L1+ IT l.	%PD-L1+ ST l.
%PD-L1 t.c.	0,631***	0,432***	.	.	.	.	.	.	0,609**	0,598**
%PD-L1+ IT l.		0,420***		.		.		.		0,532**

* - p<0,05, ** - p<0,01, *** - p<0,001

✦ - empty fields occur when one variable is a constant or with missing values

Table 17: *Correlation between PD-L1 expression in tumor and immune cells (with intratumoral and stromal localization) - %PD-L1 t.c., %PD-L1+ IT L., %PD-L1+ ST L. and amount (concentration) and the localization (intratumor and stromal) of TIL subtypes - IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3, the expression of CTLA-4 in tumor and immune cells (with intratumoral and stromal localization) - % CTLA4 t.c., %CTLA4+ IT l., %CTLA4+ ST l.*

Indicators	***Whole sample***			**Subtype LumA**			**Subtype LumB**			**Subtype HER2**			**Subtype TN**		
	%PD-L1 t.c.	**%PD-L1+ IT l.**	**%PD-L1+ ST l.**	**%PD-L1 t.c.**	**%PD-L1+ IT l.**	**%PD-L1+ ST l.**	**%PD-L1 t.c.**	**%PD-L1+ IT l.**	**%PD-L1+ ST l.**	**%PD-L1 t.c.**	**%PD-L1+ IT l.**	**%PD-L1+ ST l.**	**%PD-L1 t.c.**	**%PD-L1+ IT l.**	**%PD-L1+ ST l.**
IT CD3	0,291**	0,218*	0,339***	0,102	.	.	.	.	0,186	.	.	0,260	0,463*	0,301	0,464*
ST CD3	-0,002	0,122	0,024	0,196	.	.	.	.	0,350	.	.	0,180	-0,128	0,225	-0,389*
IT CD4	0,120	0,062	0,188*	0,327	.	.	.	.	0,257	.	.	0,021	0,314	0,201	0,514**
ST CD4	0,024	0,150	0,152	0,044	.	.	.	.	0,459*	.	.	0,029	0,045	0,299	0,066
IT CD8	0,338**	0,243*	0,269**	0,127	.	.	.	.	0,186	.	.	-0,301	0,630**	0,413*	0,627**
ST CD8	-0,098	0,100	0,028	-0,127	.	.	.	.	0,178	.	.	0,036	-0,226	0,142	-0,140
IT CD20	0,237*	0,153	0,204*	0,230	.	.	.	.	0,210	.	.	0,166	0,449*	0,302	0,384*
ST CD20	0,011	0,074	-0,033	-0,060	.	.	.	.	0,120	.	.	-0,147	0,024	0,096	-0,112
IT FP3	0,211*	0,156	0,332**	0,181	.	.	.	.	0,378	.	.	0,301	0,414*	0,282	0,504**
ST FP3	-0,031	0,146	0,239*	-0,075	.	.	.	.	0,538**	.	.	0,345	0,007	0,301	0,191
% CTLA4 t. c.	-0,129	-0,130	-0,248**	0,057	.	.	.	.	-0,245	.	.	0,012	-0,309	-0,208	-0,291
%CTLA4+ IT l.	0,513***	0,812***	0,341**	.	.	.	.	.	.	.	.	.	0,487*	0,799***	0,425*
%CTLA4+ ST l.	-0,118	-0,088	-0,226*	0,177	.	.	.	.	-0,395*	.	.	0,031	-0,270	-0,088	-0,169

* - p<0,05, ** - p<0,01, *** - p<0,001

✦ - empty fields occur when one variable is a constant or with missing values

7. The correlation analysis between the expression of CTLA-4 in tumor and immune cells (with intratumoral and stromal localization; low and high concentration) - %CTLA4 t.c., %CTLA4+ IT l., %CTLA4+ ST l. showed that in the whole sample and for the four molecular subtypes, there was a statistically reliable, right-proportional correlation only between %CTLA4 t.c. and %CTLA4+ ST t., with subtype LumA it being the strongest, and in the entire sample and the other three subtypes – less pronounced by strength (**Table 18**)

8. Correlation between the expression of CTLA-4 in tumor and immune cells (with intratumoral and stromal localization; low and high concentration) - %CTLA4 t.c., %CTLA4+ IT l., %CTLA4+ ST l., **and the other studied immune parameters** (IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3; TILs%) in the studied cases (n=100) and in those distributed by groups (individual molecular subtypes of BC – LumA, LumB, HER2, TN, each with n=25);

8.1. The correlation analysis between CTLA-4 expression in tumor and immune cells and TIL subtypes - IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3 showed, statistically significant correlations in the whole sample and for LumA, HER2 and TN subtypes (**Table 19**):

- Across the entire sample, % CTLA4 t.c. correlated weakly and cross-directionally with IT CD4; % CTLA4+ IT l. correlated weakly and unidirectionally with ST FP3; % CTLA4 + ST l. correlated weakly and inversely with IT CD4;
- In the group of subtype LumA, % CTLA4 t.c. and % CTLA4 + ST l. correlated inversely proportionally and expressed in strength with ST CD20;
- In the group of subtype HER2, %CTLA4 t.c. correlated strongly and inversely with IT CD4, and unidirectionally and moderately with ST CD8; %CTLA4 + ST l. correlated inversely and moderately in strength with IT CD4;
- In the TN subtype group, only %CTLA4 + IT l. correlated moderately and unidirectionally with ST FP3.

Table 18: *Correlation between the expression of CTLA-4 in tumor and immune cells (with intratumoral and stromal localization) - %CTLA4 t.c., %CTLA4+ IT l., %CTLA4+ ST l.*

Indicators	*Whole sample*		Subtype LumA		Subtype LumB		Subtype HER2		Subtype TN	
	%CTLA4+ IT l.	%CTLA4+ ST l.	%CTLA4+ IT l.	%CTLA4+ ST l.	%CTLA4+ IT l.	%CTLA4+ ST l.	%CTLA4+ IT l.	%CTLA4+ ST l.	%CTLA4+ IT l.	%CTLA4+ ST l.
% CTLA4 t.c.	-0,105	0,662***	.	0,790***	.	0,605**	.	0,563**	-0,166	0,536**
%CTLA4+ IT l.		-0,043		.		.		.		0,000

* - p<0,05, ** - p<0,01, *** - p<0,001 ✦ - empty fields occur when one variable is a constant or with missing values

Table 19: *Correlation between CTLA-4 in tumor and immune cells (with intratumoral and stromal localization; low and high concentration) - %CTLA4 t.c., %CTLA4+ IT l., %CTLA4+ ST l. and amount (concentration) and localization (intratumoral and stromal) of the TIL subtypes - IT CD3, ST CD3, IT CD4, ST CD4, IT CD8, ST CD8, IT CD20, ST CD20, IT FP3, ST FP3*

Indicators	*Whole sample*			Subtype LumA			Subtype LumB			Subtype HER2			Subtype TN		
	% CTLA4 t.c.	% CTLA4 + IT l.	% CTLA4 + ST l.	% CTLA4 t.c.	% CTLA4 + IT l.	% CTLA4 + ST l.	% CTLA4 t.c.	% CTLA4 + IT l.	% CTLA4 + ST l.	% CTLA4 t.c.	% CTLA4 + IT l.	% CTLA4 + ST l.	% CTLA4 t.c.	% CTLA4 + IT l.	% CTLA4 + ST l..
IT CD3	-0,042	0,131	-0,023	-0,112	.	-0,087	0,216	.	0,056	0,094	.	0,221	0,014	0,158	-0,049
ST CD3	-0,067	0,099	-0,029	-0,370	.	-0,278	0,029	.	0,097	0,084	.	0,184	0,342	0,180	0,076
IT CD4	-0,197*	0,008	-0,259**	-0,299	.	-0,271	0,053	.	-0,189	-0,541**	.	-0,436*	0,115	0,060	-0,117
ST CD4	-0,053	0,176	-0,090	-0,270	.	-0,256	-0,268	.	-0,375	0,086	.	0,000	0,255	0,344	0,255
IT CD8	-0,040	0,159	-0,067	-0,125	.	-0,039	-0,069	.	0,012	0,264	.	0,068	-0,110	0,253	-0,230
ST CD8	0,107	0,174	-0,026	0,006	.	-0,039	0,138	.	-0,012	0,439*	.	0,119	0,043	0,296	-0,080
IT CD20	-0,039	0,124	-0,021	-0,096	.	-0,035	-0,201	.	-0,131	0,165	.	0,013	-0,115	0,241	0,117
ST CD20	-0,100	0,116	-0,158	-0,515**	.	-0,568**	0,187	.	0,008	-0,080	.	-0,035	0,147	0,180	-0,014
IT FP3	-0,150	0,164	-0,125	-0,265	.	-0,140	0,160	.	-0,014	-0,086	.	-0,164	-0,143	0,293	-0,073
ST FP3	0,009	0,209*	-0,032	-0,244	.	-0,153	-0,190	.	-0,217	0,225	.	0,082	0,172	0,406*	0,146

* - p<0,05, ** - p<0,01, *** - p<0, 001

8.2. Comparative analysis of **CTLA-4 expression in tumor and immune cells** (with intratumoral and stromal localization) according to **%TILs** values, both in the whole sample and in the four molecular subtypes, found no significant differences.

9. Determination of a predominant subtype of lymphocytes in the examined cases with BC (n=100) and in those distributed by groups (individual molecular subtypes of BC - LumA, LumB, HER2, TN, each with n=25)

Comparative analysis of CD3 and CD20 lymphocyte subtypes found that (**Table 20**):

In the "Presence" category (defined based on the number of cases with CD3 and CD20 lymphocytes present, regardless of their concentration)

- Across the entire sample and LumA and LumB subtypes, the relative proportion of tumors with the presence of IT CD3 was significantly greater than that of IT CD20. For the other subtypes, there was no statistically significant difference between the presence of the considered subtypes of lymphocytes with intratumoral localization;
- In the stromal localization, in the entire sample and the four molecular subtypes, there was no statistically significant difference between the presence of the considered subtypes of lymphocytes;

In the "High" category (determined based on the number of cases with high IT and ST concentration for CD3 and CD20 lymphocytes)

- The relative proportion of tumors with a high IT CD3 concentration in the entire sample and in those with the TN subtype was significantly greater than that of IT CD20. In the other BC subtypes, a statistically significant difference between the considered subtypes of lymphocytes with a high intratumoral concentration was not found;
- In the entire sample and for all four molecular subtypes, the relative proportion of tumors with a high concentration of ST CD3 was statistically significantly greater than that of ST CD20.

Table 20: *Comparative analysis of CD3 and CD20 lymphocyte subtypes*

Concentration	Category	Localization	CD3		CD20		P
			n	%	n	%	
Presence	Whole sample	IT	88	88,0	57	57,0	**<0,001**
		ST	98	98,0	97	97,0	1,000
	Subtype LumA	IT	20	80,0	11	44,0	**0,020**
		ST	25	100,0	24	96,0	1,000
	Subtype LumB	IT	22	88,0	12	48,0	**0,006**
		ST	25	100,0	24	96,0	1,000
	Subtype HER2	IT	24	96,0	19	76,0	0,103

		ST	24	96,0	25	100,0	1,000
	Subtype TN	IT	22	88,0	15	60,0	0,053
		ST	24	96,0	24	96,0	0,470
High	Whole sample	IT	14	14,0	0	0,0	**<0,001**
		ST	67	67,0	18	18,0	**<0,001**
	Subtype LumA	IT	0	0,0	0	0,0	-
		ST	13	52,0	4	16,0	**0,017**
	Subtype LumB	IT	3	12,0	0	0,0	0,234
		ST	18	72,0	3	12,0	**<0,001**
	Subtype HER2	IT	4	16,0	0	0,0	0,118
		ST	18	72,0	5	20,0	**<0,001**
	Subtype TN	IT	7	28,0	0	0,0	**0,014**
		ST	18	72,0	6	24,0	**0,002**

Comparative analysis of lymphocyte subtypes CD4, CD8 and FP3 (**Table 21**) showed that:

In the "Presence" category (defined based on the number of cases with CD4, CD8 and FoxP3 lymphocytes present, regardless of their concentration)

- In the entire sample and TN subtype, the relative proportion of tumors having IT CD4 was significantly lower than those of IT CD8 and IT FP3, whose relative proportions were not statistically different from each other. For LumB and HER2 subtypes, IT FP3 was found statistically significantly more frequently than IT CD4, but not IT CD8, whose relative proportion was not statistically different from the other two. In the LumA subtype, a significantly higher percentage of tumors showed IT CD8 versus IT CD4, but not IT FP3, whose percentage was not statistically different from that of the other two;
- In the case of stromal lymphocytes, only in the entire sample was a statistically reliable higher percentage of ST CD8 compared to ST CD4, but not compared to ST FP3, whose relative share did not differ statistically from that of the other two. For the four molecular subtypes, there was no statistically significant difference between the presence of the examined subtypes of lymphocytes with stromal localization;

In the "High" category (defined based on the number of cases with a high IT or ST concentration for CD4, CD8 and FoxP3 lymphocytes)

- Statistically significant differences regarding the high concentration of CD4, CD8 and FoxP3 lymphocytes were found only in stromal lymphocytes in the whole sample and the considered molecular subtypes without LumA;
- In the whole sample and HER2 subtype, the relative share of high concentration of ST CD4 was significantly smaller than those of ST CD8 and ST FP3, whose relative shares did not differ statistically from each other;

- In the groups of LumB and TN subtypes, a significantly higher percentage of high concentration was found for ST CD8 compared to ST CD4, but not for ST FP3, whose percentage was not statistically different from the other two.

Table 21. *Comparative analysis of CD4, CD8 and FP3 lymphocyte subtypes*

Concentration	Category	Localization	CD4		CD8		FP3	
			n	%	n	%	n	%
Presence	Whole sample	IT	43	43,0[a]	78	78,0[b]	75	75,0[b]
		ST	89	89,0[a]	98	98,0[bc]	94	94,0[ac]
	Subtype LumA	IT	7	28,0[a]	18	72,0[bc]	14	56,0[ac]
		ST	22	88,0[a]	25	100,0[a]	24	96,0[a]
	Subtype LumB	IT	11	44,0[a]	18	72,0[ac]	20	80,0[bc]
		ST	23	92,0[a]	24	96,0[a]	24	96,0[a]
	Subtype HER2	IT	15	60,0[a]	22	88,0[ac]	23	92,0[bc]
		ST	22	88,0[a]	25	100,0[a]	24	96,0[a]
	Subtype TN	IT	10	40,0[a]	20	80,0[b]	18	72,0[b]
		ST	22	88,0[a]	24	96,0[a]	22	88,0[a]
High	Whole sample	IT	8	8,0[a]	8	8,0[a]	7	7,0[a]
		ST	7	7,0[a]	39	39,0[b]	28	28,0[b]
	Subtype LumA	IT	0	0,0	0	0,0	0	0,0
		ST	1	4,0[a]	7	28,0[a]	5	20,0[a]
	Subtype LumB	IT	0	0,0[a]	2	8,0[a]	1	4,0[a]
		ST	2	8,0[a]	9	36,0[bc]	6	24,0[ac]
	Subtype HER2	IT	8	32,0[a]	2	8,0[a]	3	12,0[a]
		ST	2	8,0[a]	11	44,0[b]	10	40,0[b]
	Subtype TN	IT	0	0,0[a]	4	16,0[a]	3	12,0[a]
		ST	2	8,0[a]	12	48,0[bc]	7	28,0[ac]

*- the same letters on the horizontal lines mean the absence of a significant difference, and the different ones - the presence of one ($p<0,05$)

10. Determination of preferred localization of lymphocytes in the studied cases with BC (n=100) and in those distributed by groups (individual BC molecular subtypes - LumA, LumB, HER2, TN, each with n=25)

The preferred localization - intratumoral (IT) or stromal (ST), of the lymphocyte subtypes (CD20, CD3, CD4, CD8, FoxP3, PD-L1 and CTLA4), was determined based on the number of cases with stromal/intratumoral localization of the lymphocyte infiltrate regardless of the concentration of cells (category "presence") and at their high concentration (category "high concentration").

From **Tables 22-26** it is clear that:

- In the entire sample, there was the highest number of indicators (almost all) where the difference studied is statistically reliable, with stromal localization being a more common site for the spread of the immune cell infiltrate. Only for CD3 and CD4 with a high concentration was the difference statistically negligible;

Subtype LumA, category **"presence"**

- Stromal localization was significantly preferred for CD4, CD8, CD20, FoxP3 and CTLA4 positive lymphocytes, regardless of their concentration;

Subtype LumA, Category **"high concentration"**

- Stromal localization was statistically significantly more common with CD3, CD8 and CTLA4. Intratumoral localization with a high concentration was absent in all considered indicators;

Subtype LumB, category **"presence"**

- Stromal localization was statistically significantly preferred for CD3, CD4, CD20, PD-L1 and CTLA4;

Subtype LumB, category **"high concentration"**

- Stromal localization was significantly higher for CD3 and CD8;

Subtype HER2, category **"presence"**

- Stromal localization was statistically significantly more frequent for the high concentration of CD8, CD20 and CTLA4 lymphocytes;

Subtype HER2, category **"high concentration"**

- Stromal localization was statistically significantly more common with CD3, CD8 and CTLA4;

Subtype TN, category **"presence"**

- Stromal localization was significantly higher for CD4, CD20 and CTLA4;

Subtype TN, category "**high concentration**"

- Stromal localization was significantly higher for CD3, CD8, CD20, PD-L1 and CTLA4.

Table 22. *Comparative analysis of the preferred localization by lymphocyte subtypes (CD20, CD3, CD4, CD8 and FoxP3, PD-L1 and CTLA4 (whole sample)*

Concentration	Localization / Indicator	Intratumoral		Stromal		P
		n	%	n	%	
Presence	CD3	88	88,0	98	98,0	**0,013**
	CD4	43	43,0	89	89,0	**<0,001**
	CD8	78	78,0	98	98,0	**<0,001**
	CD20	57	57,0	97	97,0	**<0,001**
	FoxP3	75	75,0	94	94,0	**<0,001**
	PD-L1	3	3,0	18	18,0	**0,001**

	CTLA4	2	2,0	50	50,0	**<0,001**
High	CD3	18	72,0	67	67,0	0,539
	CD4	14	14,0	7	7,0	0,160
	CD8	8	8,0	39	39,0	**<0,001**
	CD20	0	0,0	18	18,0	**<0,001**
	FoxP3	7	7,0	28	28,0	**<0,001**
	PD-L1	0	0,0	6	6,0	**0,038**
	CTLA4	0	0,0	33	33,0	**<0,001**

Table 23. *Comparative analysis of the preferred localization by lymphocyte subtypes (CD20, CD3, CD4, CD8 and FoxP3, PD-L1 and CTLA4 (LumA subtype)*

Concentration	**Localization / Indicator**	**Intratumoral**		**Stromal**		**P**
		n	%	n	%	
Presence	CD3	20	80,0	25	100,0	0,059
	CD4	7	28,0	22	88,0	**<0,001**
	CD8	18	72,0	25	100,0	**0,014**
	CD20	11	44,0	24	96,0	**<0,001**
	FoxP3	14	56,0	24	96,0	**0,003**
	PD-L1	0	0,0	0	0,0	-
	CTLA4	0	0,0	18	72,0	**<0,001**
High	CD3	0	0,0	13	52,0	**<0,001**
	CD4	0	0,0	1	4,0	1,000
	CD8	0	0,0	7	28,0	**0,014**
	CD20	0	0,0	4	16,0	0,118
	FoxP3	0	0,0	5	20,0	0,059
	PD-L1	0	0,0	0	0,0	-
	CTLA4	0	0,0	13	52,0	**<0,001**

Table 24: *Comparative analysis of preferential localization by lymphocyte subtypes (CD20, CD3, CD4, CD8 and FoxP3, PD-L1 and CTLA4 (LumB subtype)*

Concentration	**Localization / Indicator**	**Intratumoral**		**Stromal**		**P**
		n	%	n	%	
Presence	CD3	22	88,0	25	100,0	**0,013**
	CD4	11	44,0	23	92,0	**<0,001**
	CD8	18	72,0	24	96,0	0,054
	CD20	12	48,0	24	96,0	**<0,001**
	FoxP3	20	80,0	24	96,0	0,192
	PD-L1	0	0,0	6	24,0	**0,030**
	CTLA4	0	0,0	9	36,0	**0,003**
High	CD3	3	12,0	18	72,0	**<0,001**
	CD4	0	0,0	2	8,0	0,470
	CD8	2	8,0	9	36,0	**0,040**
	CD20	0	0,0	3	12,0	0,234
	FoxP3	1	4,0	6	24,0	0,103
	PD-L1	0	0,0	0	0,0	-

	CTLA4	0	0,0	5	20,0	0,059

Table 25. *Comparative analysis of the preferred localization by lymphocyte subtypes (CD20, CD3, CD4, CD8 and FoxP3, PD-L1 and CTLA4 (HER2 subtype)*

Concentration	**Localization / Indicator**	**Intratumoral**		**Stromal**		**P**
		n	%	n	%	
Presence	CD3	24	96,0	24	96,0	0,470
	CD4	15	60,0	22	88,0	0,053
	CD8	22	88,0	25	100,0	**0,013**
	CD20	19	76,0	25	100,0	**0,030**
	FoxP3	23	92,0	24	96,0	1,000
	PD-L1	0	0,0	2	8,0	0,470
	CTLA4	0	0,0	14	56,0	**<0,001**
High	CD3	4	16,0	18	72,0	**<0,001**
	CD4	8	32,0	2	8,0	0,077
	CD8	2	8,0	11	44,0	**0,010**
	CD20	0	0,0	5	20,0	0,059
	FoxP3	3	12,0	10	40,0	0,053
	PD-L1	0	0,0	0	0,0	-
	CTLA4	0	0,0	8	32,0	**0,007**

Table 26. *Comparative analysis of the preferred localization by lymphocyte subtypes (CD20, CD3, CD4, CD8 and FoxP3, PD-L1 and CTLA4 (subtype TN)*

Concentration	**Localization / Indicator**	**Intratumoral**		**Stromal**		**P**
		n	%	n	%	
Presence	CD3	22	88,0	24	96,0	0,602
	CD4	10	40,0	22	88,0	**0,001**
	CD8	20	80,0	24	96,0	0,192
	CD20	15	60,0	24	96,0	**0,006**
	FoxP3	18	72,0	22	88,0	0,289
	PD-L1	3	12,0	10	40,0	0,053
	CTLA4	2	8,0	9	36,0	**0,040**
High	CD3	7	28,0	18	72,0	**0,005**
	CD4	25	100,0	2	8,0	0,470
	CD8	4	16,0	12	48,0	**0,034**
	CD20	0	0,0	6	24,0	**0,030**
	FoxP3	3	12,0	7	28,0	0,289
	PD-L1	0	0,0	6	24,0	**0,030**
	CTLA4	0	0,0	7	28,0	**0,014**

11. Analysis of PD-L1 and CTLA-4 tumor and immune expression in individual BC subtypes - LumA, LumB, HER2 and TN.

The analysis performed for the expression of PD-L1 and CTLA-4 in the tumor (% PD-L1 t.c., % CTLA4 t.c.) and stromal/intratumoral immune cells (% PD-L1+ ST l., % CTLA4+ ST l., %PD-L1+ IT l., %CTLA4+ IT l.), was based on the total number of cases with positivity (regardless of their concentration - "presence" category) and at their high concentration ("high concentration" category).

It was found that (**Table 27**):

In the category **"presence"**

- There were significant differences in the indicators %PD-L1 t.c., %PD-L1+ ST l., %CTLA4 t.c. and %CTLA4+ ST l.;

- Regarding %PD-L1 positivity in t.c. statistically significantly higher percentage had the patients with TN subtype compared to the LumB and HER2 subtypes, but not compared to those with the LumA subtype, whose relative share did not differ statistically from those of the other subtypes;
- Again, in the TN subtype we observed statistically significantly the highest percentage of %PD-L1+ ST l. this time relative to subtypes LumA and HER2, but not relative to the relative share of subtype LumB;
- At %CTLA4 t.c., patients of the LumA subtype had a statistically significantly higher percentage compared to the LumB and TN subtypes, but not compared to those with the HER2 subtype, whose relative share was significantly higher only than that of those with the LumB subtype;
- Regarding %CTLA4+ ST l. they had a statistically significantly higher percentage LumA subtype patients versus LumB and TN subtypes, but not versus those with the HER2 subtype, whose relative proportions did not differ statistically from those of the other subtypes;

In the category **"High concentration"**

- There are significant differences in the indicators %PD-L1+ ST l., %CTLA4 t.c. and %CTLA4+ ST l.;

- Regarding %PD-L1 + ST l. there was a statistically significantly higher percentage in subtype TN compared to all other subtypes, whose relative shares did not differ statistically from each other;
- At %CTLA4 t.c., they had a statistically significantly higher percentage the LumA subtype patients versus all other subtypes, whose relative proportions were not statistically different from each other;

- Regarding %CTLA4 + ST l. – patients with LumA subtype compared to LumB subtype had a significantly higher percentage, but not compared to those with HER2 and TN subtypes, whose relative shares did not differ statistically, both among themselves and from those of the remaining subtypes.

Table 27. *Comparative analysis of PD-L1 and CTLA-4 expressions (presence and high) in different molecular subtypes*

Concentration	Indicator	Subtype LumA		Subtype LumB		Subtype HER2		Subtype TN	
		n	%	n	%	n	%	n	%
Presence	%PD-L1 t.c.	1	4,0^{ac}	0	0,0^{a}	0	0,0^{a}	6	24,0^{bc}
	%PD-L1+ IT l.	0	0,0^{a}	0	0,0^{a}	0	0,0^{a}	3	12,0^{a}
	%PD-L1+ ST l.	0	0,0^{a}	6	24,0^{bc}	2	8,0^{ac}	10	40,0^{b}
	%CTLA4 t.c.	15	60,0^{a}	4	16,0^{b}	12	48,0^{ac}	6	24,0^{bc}
	%CTLA4+ IT l..	0	0,0^{a}	0	0,0^{a}	0	0,0^{a}	2	8,0^{a}
	%CTLA4+ ST l.	18	72,0^{a}	9	36,0^{bc}	14	56,0^{ac}	9	36,0^{bc}
High	%PD-L1 t.c.	0	0,0^{a}	0	0,0^{a}	0	0,0^{a}	1	4,0^{a}
	%PD-L1+ IT l.	0	0,0	0	0,0	0	0,0	0	0,0
	%PD-L1+ ST l.	0	0,0^{a}	0	0,0^{a}	0	0,0^{a}	6	24,0^{b}
	% CTLA4 t. c.	6	24,0^{a}	0	0,0^{b}	0	0,0^{b}	0	0,0^{b}
	%CTLA4+ IT l.	0	0,0	0	0,0	0	0,0	0	0,0
	%CTLA4+ ST l.	13	52,0^{a}	5	20,0^{bc}	8	32,0^{ac}	7	28,0^{ac}

* - the same letters on the horizontal lines mean the absence of a significant difference, and the different ones - the presence of one ($p<0,05$)

V. DISCUSSION

The results of our study were similar to the ECIS data from 2020, regarding the age-specific incidence of BC in Bulgaria. In our study population, the incidence of this disease increases sharply after the age of 30, reaching its peak in the age group of 70-79 year old women, and then decreases.

Regarding the age distribution of BC subtypes, we found that the LumA subtype is more common in younger patients, compared to the other variants of this neoplasm, which are mostly diagnosed after 50 years of age. Our results differ from those obtained in most other studies. According to them, young age (which has no clear definition and is defined differently in individual studies, including ≤35/≤40 years of age) is an independent prognostic factor for more frequent occurrence of biologically and clinically aggressive tumors. According to data from some studies, HER2 positive and TN prevail at an earlier age, incl. basal-like subtype and with BRCA mutations, and according to others, the luminal B subtype is more common in young patients. It has also been found that even a luminal A variant diagnosed in cases at an earlier age has a more aggressive clinical course compared to that demonstrated in older patients. On the other hand, there is evidence that luminal variants have an age-independent prognostic value, with the Luminal A subtype being associated with the most favorable clinical outcome compared to the other molecular subtypes of BC. Further studies are needed to more precisely determine the significance of age at the onset of individual subtypes of carcinomas, its association with additional characteristics of carcinomas, and its impact on patient prognosis.

Taking into account the distribution of BC subtypes in terms of 5-year overall survival, our data confirmed the findings of other studies that the LumA subtype is characterized by the best prognosis, and the TN and HER2-positive non-luminal subtypes are the least favorable, as LumB occupies an intermediate position. It should be noted that the patients we studied with the HER2 positive subtype were diagnosed in a period when neoadjuvant therapy was not a preferred therapeutic approach. To date, this type of treatment has proven beneficial in patients with HER2-positive BC, achieving a complete pathological response in a large percentage of cases, improving their prognosis. Also, BC has a proven biological heterogeneity, and the individual surrogate IHC-established variants do not fully reflect the molecular classification and include different subtypes. This affects the clinicopathological characteristics of BC. The need arises to find new prognostic and predictive markers complementing the surrogate classification. Their detection may allow a more accurate stratification of patients, related to their more precise and long-term prognosis, which will enable the application of a personalized therapeutic approach.

In our study, additional favorable prognostic factors were again found predominantly in LumA tumors compared to the other BC subtypes. With the LumA variant, patients are more often diagnosed in clinical stage I, with tumor

size ≤3 cm, which is highly differentiated - G1, mostly without lymphovascular invasion and without metastatic spread in axillary lymph nodes. Unfavorable clinico-pathological factors such as advanced clinical stage (III and IV), larger tumor size (>3 cm), presence of LVI and lymphogenous spread in LN, are found mostly in LumB and HER2 subtypes positive non-luminal BC, and the high histological grade – G3, mainly in HER2 positive and TN subtype. The intermediate position of the TN variant in relation to some of the stated indicators confirms its features described in the literature, e.g. the rare metastasis by lymphogenous route. Although some of the patients with this subtype are diagnosed at an earlier clinical stage (mostly in the II) with lesions of smaller sizes, the low degree of differentiation of the tumors, in addition to the shorter survival of the patients suggest the aggressive biological features of part of TN carcinomas. A possible reason for them is the basal-like molecular characteristics of these neoplasms. In accordance with other studies, in our study we found the highest frequency of the basal-like subtype precisely in TNBC, mainly with a special morphological type. In a smaller percentage, this variant was also determined in the other subtypes of BC, which probably contributes to their less favorable clinical behavior. In our study, the basal subtype was associated with a poor prognosis, with patients with this subtype more often having <5 - year overall survival. Establishing the basal-like phenotypic manifestations in BC may allow the selection of such patients, with a different surrogate molecular subtype, in which to apply a personalized "more aggressive" and combined therapeutic approach.

Another pathological factor studied was the histological variant of the carcinoma, which may also have prognostic value. In our study, NST carcinomas predominated, most of which were included in the HER2 subtype category, and lobular carcinomas had the smallest relative proportion, being predominantly LumB subtype. Some of the subtypes of the histological type, in addition to their degree of differentiation, can have both positive and negative effects on the clinical outcome of patients. We also found that a greater proportion of the special BC types we included were in the LumA and TN categories. In accordance with previous researches, in our study in the LumA group BC were special types with a favorable prognosis - incl. mucinous and tubular type. On the other side was the TN subtype, comprising the heterogeneous group of the metaplastic type, with an unfavorable prognosis for most of its variants. In addition to it, in the studied category of TNBC was also a medullary type, usually with a positive clinical result, despite its characteristic low degree of differentiation.

The established data show that the prognosis for BC subtypes is a complex and complicated indicator, and the classic prognostic factors are not sufficiently unambiguous and categorical. More than one clinicopathological factor needs to be taken into account for a more accurate determination of the clinical outcome in patients with BC. The need to find new prognostic biomarkers is also confirmed.

In recent years, features of the tumor microenvironment have been studied as such, incl. various factors of antitumor IR. Among them, TILs are one of the most attractive indicators and are included in most studies.

There are still conflicting data on their prognostic and predictive value in BC patients. According to some studies, only a high concentration of TILs is clinically significant, and as such a TILs >50% is accepted. A preponderance of results show a directly proportional relationship between the degree of TILs infiltration and the response to applied therapy. Their positive influence on the prognosis in patients with BC is also more often reported. In addition, a difference in immune activity has been found among BC subtypes, with the HER2 positive and TN variants most often defined as the most immunogenic, with a more pronounced immune cell infiltrate.

In our study, a predominant percentage of cases had a TILs ≤50%, with the highest relative proportion for the LumA subtype, in which no tumor was found with a TILs >50%. LPBC - with TILs >50%, was found in a smaller proportion of patients studied, mainly with HER2 subtype, followed by TNBC, which is consistent with most studies.

Correlation of TILs quantitative values with proven prognostically significant clinicopathological factors may contribute to a better understanding of the clinical significance of the immune infiltrate.

According to the data of our study, a high concentration of stromal lymphocytes is associated with unfavorable clinicopathological factors. TILs >50% was found only in carcinomas with a low degree of differentiation - G3 and mainly in patients with metastatic LN. In addition, high TILs concentration was associated with < 5 year overall survival in the studied patients.

Our results contradict those of most other studies. This warrants a more thorough study of the immune response in BC, not only considering the concentration of lymphocytes, but also subtyping the lymphocyte infiltrate.

Different types of immune cells have been found to perform different and specific functions, interacting both with each other and with non-lymphoid cells. It is also suggested that lymphocyte subtypes have different involvement in IR in individual BC subtypes. In order to determine the specific role of the immune infiltrate in BC, its composition, preferred localization and degree of infiltration in the tumor area are increasingly being studied.

In our study, cases with T-cell CD3-expression were found to predominate compared to those positive for CD20 B-lymphocytes (regardless of the semiquantitative values of the indicated cell subtypes). This dependence applies to the intratumoral localization of the cells, and for the stromal no statistically significant difference was found for the subtyped lymphocytic infiltrate. However, a high concentration of CD3+ T-lymphocytes was more common than a high degree of infiltration with CD20+ B-lymphocytes not only intratumorally, but also stromally.

Of the T lymphocyte subtypes, a greater number of cases with intratumoral and stromal cytotoxic CD8+ and regulatory FoxP3+ cells were present in the entire sample, with a slight predominance of CD8+ lymphocytes in both locations. Regarding the high concentration of T cell subtypes, a significant difference was found only in their stromal infiltration. In the stroma, a high number of CD4+ T-helpers is less often reported, compared to the other two types of T-cells. No predominant T-lymphocyte subtype was found with high concentration in intratumoral localization.

In the BC cases studied by us and in the four molecular subtypes, most types of lymphocytes studied, involved in humoral and cellular IR (CD20+ B-, CD3+ T- and T-lymphocyte subtypes - cytotoxic CD8+ and regulatory FoxP3+), mainly infiltrate the stroma (as in both low and high). A statistically reliable dependence was established in the majority of the categories of the comparative analysis. Only for the high concentration of helper CD4+ T-cells, only a trend for preferential intratumoral spread was observed, mainly for the HER2 and TNBC subtypes.

In our study, low concentrations of the studied lymphocyte subtypes (CD20+B, CD3+ T, CD8+ T-cytotoxic, FoxP3+ T-regulatory and CD4+ T-helper) predominated intratumorally, compared to their high values. CD4 and CD20 cells were the ones that rarely infiltrate the tumor areas in BC, because in the largest percentage of cases no such type of positive cells were observed in this localization, compared to the other subtypes of lymphocytes. In addition, the high intratumoral concentration was mainly found in CD3 T-lymphocytes and the T-cell subtypes – CD4, CD8 and FoxP3, and it was not characteristic of CD20 B-lymphocytes.

Throughout the sample, in terms of stromal localization, with the exception of CD3+ T - cells, low versus high concentrations of the studied immune cell subtypes again prevailed. Of the lymphocyte subtypes, CD4 T-helpers were the ones that most often did not infiltrate the stroma in the BC cases studied. On the other hand, the high amount in stromal location was mostly characteristic of CD3 T-lymphocytes and was found more often in some of their subtypes – CD8+ cytotoxic and FoxP3 regulatory cells.

Of the BC subtypes studied, high lymphocyte concentrations were mostly observed in TN and HER2 positive non-luminal variant. Regarding the intratumoral localization, the TN subtype was preferred for the high concentration of CD3 and CD8, HER2 for CD4, and FoxP3 was found to an equal extent in these BC variants, compared to the luminal ones. Although high intratumoral CD20 concentrations were not reported in any case, low numbers were most characteristic of the HER2 subtype.

Again, TN and HER2 positive BC were the subtypes in which a high stromal concentration of lymphocytes is most often reported, but for some cell subtypes it is found equally with the LumB variant. TNBC is a preferred subtype for high concentration of stromal CD8 and CD20, HER2 for FoxP3, and high

stromal CD3 and CD4 counts were equally distributed among LumB, HER2 positive and TNBC subtypes.

The above results were consistent with those obtained in other studies. We confirm the data that TN and HER2 positive BC are more immunogenic subtypes compared to luminal. However, according to our study, there was probably a subpopulation in the LumB variant that also stimulates a pronounced antitumor IR.

Consistent with data from other studies, we found that the stroma is a preferred site of infiltration by most types of lymphocytes. However, immune cells with different localization have been suggested to have different biological and clinical significance. Although less frequently detected, intratumoral lymphocytes probably more accurately reflect immune activity in the tumor area due to their direct interaction with tumor cells. According to the results of some studies, the concentration of these lymphocytes is irrelevant to the final effect of their action. Important are the mechanisms that are activated during intercellular contacts - inhibitory or activating.

Similar to other studies, our results indicate that T-cell-mediated immunity was predominant in the antitumor response in BC, and humoral B-cell has a secondary role. Also, cytotoxic CD8+ T-lymphocytes are a predominant T-cell subtype. Their activation is possible with sufficient immunogenic stimulation, which would allow the creation of an effective antitumor IR. The observed tendency for predominant intratumoral localization by CD4+ T - cells, especially in the more immunogenic variants BC - HER2 positive and TN, probably indicates an attempt to support the activation of the effector population of T - lymphocytes. On the other hand, however, it may reflect inhibitory immune activity, since the main representatives of T-cell immunosuppressors - FoxP3 T-regulatory cells, were also positive for the CD4+ molecule and were found more often in these subtypes of BC.

Our results for a predominant TIL subtype were at variance with those obtained by some other studies. Possible reasons for the inconsistencies in the literature data were differences in the design of the respective studies (heterogeneous selection of tumor subtypes, examination of unequal numbers and different markers of the lymphocyte infiltrate, use of different techniques for establishing TILs and methods for evaluating immunophenotyped cells).

The different quantity, composition, and preferred localization of lymphocyte subtypes likely reflect unequal cell biology in carcinomas, with heterogeneous capabilities to effectively control tumor progression and engage different mechanisms to generate immunological memory. Establishing meaningful correlations between immune cells and their specific features is an important functional aspect of antitumor immunity. Finding them may contribute to a better prognosis in BC and provide guidance for the creation of new immunotherapeutic targets.

Statistically significant correlations were found between the individual subtypes of the lymphocyte infiltrate both in the studied population of patients with BC and among the individual surrogate subtypes of this neoplasm.

In the examined cases with BC and from the subtypes - in TN and luminal variants, CD20+ B lymphocytes correlate with all subtypes of T-lymphocytes in intratumoral localization and only with FoxP3 Treg cells in stromal infiltration. It is suggested that the established dependence reflects a complementary/auxiliary role of B-cells in performing T-cell IR, mainly in the intratumoral area of BC.

According to our study, CD8-Tc cells are the predominant subtype of CD3+ T-lymphocytes, with established correlations between them in all four molecular subtypes of BC. Intratumoral CD8-Tc and FoxP3-Treg TIL subtypes were shown to occur more frequently together in the studied cases, mainly in the TN subtype and to a lesser extent in the luminal subtypes.

Stromal lymphocytes are a more heterogeneous population, with CD8 Tc cells again showing a tendency for a predominant T-cell subtype, with established correlations between CD3+ and CD8+ stromal cells in the studied tumors and of the BC subtypes - in the luminal variants. Again, a dependence was observed between the same subtypes of T lymphocytes - CD8-Tc and FoxP3-Treg in the stroma of the entire studied population and in that of TN and LumB subtype.

It is possible that the high amount of cytotoxic CD8-Tc cells is a signal of an active attempt by the IS to reject the tumor, which, however, is unsuccessful due to their suppression by the suppressor FoxP3+ Treg cells. A suppressive effect on the part of tumor cells is also allowed, which is specific for individual molecular subtypes of BC. According to data from some studies, other TIL subtypes are predominant and interrelated. One possible reason is again a difference in study design.

It's proved that Foxp3-positive cells have been shown to represent a subtype of Treg CD4+ T – lymphocytes. Consistent with these data, we found an association between stromal and intratumoral CD4+ T and FoxP3+ Treg in the study cohort of BC patients. Such a correlation was found only in some of the subtypes of BC – with HER2 and LumB for the stromal cells and with HER2 and TN for the intratumorally counted lymphocytes. It is likely that FoxP3 regulatory cells have a different involvement in tumorigenesis in different types of BC and are involved in IR only in some of them.

A weak correlation was also found between intratumoral CD4 and CD8 in the general study population and a strong correlation only in the TN subtype. CD4 + TILs have been shown to be sufficient to eliminate tumor cells in the absence of CD8+ T cells in some tumors. More often, however, both cell lines (CD4+ and CD8+) are required for effective tumor removal. The role of some CD4+ TIL subtypes in the antitumor response is often activation of CD8 + TILs, leading to tumor destruction by CD8+ cytotoxic T lymphocytes. It is possible that the simultaneous presence (in the absence of the FoxP3 subtype) of intratumoral CD4 and CD8 T lymphocytes reflects a more favorable prognosis for these neoplasms.

Finding correlations between specific features of the lymphocytic infiltrate with proven prognostically significant clinicopathological indicators may provide a clearer picture of the role of the immune cell response we observed in BC. Also, establishing these relationships is part of the complex process of incorporating a biomarker into routine practice.

In the whole sample and in TN subtype G3 carcinomas had a high concentration of IT CD3. It was also found that the studied cases with G3 more often reported a low concentration of IT CD4. In addition to these data, in the low degree of differentiation, FoxP3 ITs were more often present, regardless of their concentration (low or high). A high concentration of ST FoxP3 cells was also found to predominate in G3 tumors, and this correlation was particularly characteristic of LumB carcinomas.

From the above data, it can be summarized that in BC, intratumoral FoxP3 T-regulatory cells, regardless of their quantity, are an unfavorable prognostic factor when combined with a low concentration of intratumoral CD4 helper T-lymphocytes. However, the high degree of infiltration with regulatory T cells in the stroma negatively affects the prognosis only in the Lum B variant. We confirm the results of other studies that identify FoxP3 as a potential marker for cancer progression.

In the whole studied population and in the patients with TN subtype with histologically proven other morphological (special) type, a high concentration of IT CD8+ was more often reported. In this group of histological variants, cases without IT FoxP3 predominated. In TN carcinomas with NST histological type, a predominantly low concentration of IT CD8+ subtype lymphocytes was found, with no significant differences regarding IT FoxP3. No significant association with TIL subtypes was reported in lobular carcinoma.

In patients with HER2 positive carcinomas with presence of LVI, a high concentration of ST CD8 is more often found, and in those without such invasion, their low concentration prevails.

The high concentration of ST CD20 is associated with the presence of LVI in the studied cases with BC and with metastatic LN in TNBC.

In patients with >5 years of overall survival for HER2 subtype, IT CD20 were found to be more often absent, and in shorter survival, their concentration was found to be low (considering that a high concentration of this cell type was not reported in none of the studied cases).

Tumors ≤3 cm in size in the LumA subtype mostly have a low concentration of IT CD8, and in larger tumors such cells are absent, and their high concentration is not observed at all in this BC subtype.

According to data from some studies, the prognostic value of cytotoxic T cells is controversial in BC subtypes and depends on the localization of the cells. As a summary of our results, it can be said that CD8+ T - lymphocytes with intratumoral localization are associated with a positive effect in LumA tumors of small size. However, the high concentration of this TIL subtype in the stroma of

HER2 carcinomas probably acts as an unfavorable prognostic factor. Intratumoral CD8+ positive T cells may effectively perform their cytotoxic function in most histological types of carcinomas, but mostly with another morphological type and in combination with the absence of regulatory cells in the same area of the neoplasm. In addition, cytotoxic T lymphocytes were not prognostic in NST and lobular carcinoma subtypes. It is possible that it depends on other factors in these histological variants.

Consistent with most older studies, B lymphocytes were associated with an unfavorable prognosis, with HER2-positive BC being negatively influenced by IT CD20, and in TN subtype by high concentration of ST CD20. However, we are at odds with data from a meta-analysis of more recent studies, according to which B cells have a predominantly protective rather than harmful effect in BC.

In addition to the above results, a difference was found in the reported amount of certain types of immune cells when examining biopsy samples from different surgical interventions (mastectomy or excision). A higher concentration of ST CD3, IT CD3 and IT CD4 was more often found in mastectomy compared to excisional biopsy, where their lower values predominate. It is likely that the calculation of the lymphocyte infiltrate in a larger operative material will allow a more precise calculation of IR in BC. The main difficulties in determining TILs arise from the partially represented tumor tissue in smaller biopsy materials, considering the heterogeneity of this neoplasm in terms of tumor cellularity, the amount of tumor stroma, and the different distribution pattern and composition of TILs in individual histologies and molecular variants, with varying degrees of differentiation.

The obtained results for the correlations of the phenotyped lymphocyte infiltrate in BC were not sufficient to understand their role and prognostic value. A study of the molecular mechanisms allowing tumor cells to “escape” IR will provide additional information on the clinical significance of TILs. Chief among them are those involving the PD-L1 and CTLA-4 molecules, which can be expressed in both immune and tumor cells.

Our results regarding PD-L1 and CTLA-4 positivity indicated that their expression is rarely found in neoplastic cells in the BC population studied. However, of the BC subtypes, TN is the one in which tumor expression for PD-L1 is most frequently reported, and tumor positivity for CTLA-4 is most frequently observed in LumA. For both markers, the high expression in tumor cells (PD-L1 and CTLA-4 $\geq$50%) was determined in the same BC subtypes, respectively.

According to the obtained results, PD-L1 positivity in the immune cells was reported in a low percentage of the studied cases. In their intratumoral location, a high concentration of positive cells was not found in any case, and the low concentration was found only in TNBC. In this subtype, compared to the other BC variants, stromally located PD-L1 positive immune cells, incl. with their high concentration, which is statistically reliable.

Intratumoral immune cells expressing CTLA-4 have been reported in a small number of neoplasms studied. They are found only in TN subtype, their concentration is low and there are no cases with the high degree of intratumoral infiltration of this cell population. In the stromal localization, CTLA – 4 positive immune cells, incl. with high concentration are reported most often in LumA subtype.

In addition to the above data, we found differences regarding PD-L1 expression in the categories of basal and non-basal BC cases. In the non-basal subtypes, PD-L1 positive stromal immune cells were more often absent, while in the basal subtypes, their high concentration was mostly found. Compared to non-basal neoplasms, positivity in tumor cells was also more characteristic of the basal variant of BC.

These results indicate that inhibitory mechanisms are differentially involved in different subtypes of BC. TNBC, in which the basal-like subtype of neoplasms are most often found, is preferred for the inclusion of the checkpoint suppressor pathways. In it, the main role is played by those with the participation of PD-L1, and a secondary one - with the involved CTLA-4 molecule. Our results are consistent with those obtained in other studies, according to which checkpoint molecules have a major role in TNBC. In contrast, in our study the LumA variant also stood out as one of the BC subtypes in which IR-suppressing mechanisms are more frequently involved. However, only one of the studied was found to be involved in it, the one related to the CTLA-4 molecule. It is possible that individual mechanisms have different clinical significance in BC subtypes. The possibility of cross-linking between them, with a synergistic or mutually exclusive effect in the antitumor IR, is also allowed. In order to clarify their role in the studied cases with BC, we looked for correlations between the expression of PD-L1 and CTLA-4 molecules by tumor and immune cells, both among themselves and with other immune and clinicopathological factors.

In the studied cases with BC and mainly in those with the TN subtype, a directly proportional correlation was found between the expression of PD-L1 in tumor and immune cells, and the relationship was more pronounced in intratumoral localized ones. As a subtype associated with adverse clinicopathological factors, a synergistic mechanism of action with an inhibitory effect on the involvement of the PD-L1 molecule in the tumor microenvironment is suggested.

The results of the correlation analysis for CTLA-4 show that there is a one-way relationship between the positivity for this molecule only in tumor and stromal located immune cells, mainly in LumA subtype BC. Given the proven favorable prognostic factors in this variant, we allow the possibility that in it the involvement of the CTLA-4 molecule has a "stopping" effect on the infiltration of TIL subtypes with suppressive immune activity. The lack of activity of this inhibitory mechanism is also possible due to the influence of additional immune and tumor factors.

In the studied patient population, and mainly in those with TNBC, a unidirectional and directly proportional relationship was established between the expression of PD-L1 by tumor cells and CTLA-4 positive intratumoral lymphocytes, as well as with the concentration of almost all subtypes of lymphocytes (without CD4+ T-helpers) with intratumoral localization. According to additional results, there is a one-way correlation between the expression of PD-L1 and CTLA-4 in intratumoral immune cells. There is also a significant relationship between PD-L1 positivity in immune cells and the concentration of CD3+ T- lymphocytes, predominantly the CD8+ cytotoxic subtype with an intratumoral location. In TNBC IT CTLA-4+ positive lymphocytes are unidirectionally associated with ST FoxP3.

Additionally, the assumption is confirmed that the leading inhibitory mechanism in intratumoral IR in BC, mainly in the TN subtype, is the one involving the PD-L1 molecule, with a possible complementary effect from CTLA-4. It is also possible that the found correlation of PD-L1 expression in tumor and immune cells with the increased concentration of CD8+ T lymphocytes indicates an active attempt of the body to remove the neoplastic proliferation. The observed linear increase in the number of FoxP3 T-regulatory cells (mentioned above) probably reflects the suppressed activity of effector cells. Increased numbers of intratumoral CD20+ B-lymphocytes in PD-L1 positive tumors may also contribute to the progression of TN neoplasms. In addition, it can be assumed that the amount of lymphocyte subtypes is not an independent determinant for the performance of an effective antitumor IR. Even in the presence of a high concentration of cytotoxic T-lymphocytes, the activity of the inhibitory pathways may not allow the performance of their function. It is also assumed that precisely patients with high PD-L1 expression on tumor and immune cells, in combination with a high concentration of CD8+, FoxP3 and CD20+, are most suitable for immune therapeutic modulation. In them, effective recovery of the antitumor IR is expected as a final effect.

In the studied cases with BC and in those with HER2 subtype, an inverse correlation was found between CTLA-4 positivity in tumor cells and the concentration of intratumoral CD4+T helpers and a unidirectional relationship between expression in tumor cells and the concentration of CT CD8+. There is also an inverse relationship between ST CTLA-4+ and IT CD4+ immune cells. According to these results, it can be suggested that the infiltration in the tumor area by CD4+ T-helpers and the activity of cytotoxic T-lymphocytes is suppressed mainly with the participation of the CTLA-4 checkpoint molecule in HER2 subtype BC. Modulation of this checkpoint mechanism may be a promising new avenue for the treatment of HER2+ breast cancer. Available data from some studies encourage the use of immunotherapy associated with CTLA-4 blockade, specifically in patients with HER2 positive tumors.

Additional correlations were found for luminal BC subtypes. CTLA-4 positivity in LumA tumor cells and stromal lymphocytes of this subtype correlated

inversely with the concentration of ST CD20+ B- lymphocytes. It is possible that the obtained result reflects a positive influence of CTLA-4 in this variant of carcinoma, given the favorable prognosis in the studied group of patients and the negative effect of B cells in BC established by us and in other studies.

In the patients studied by us and mainly in the LumB subtype, an inverse relationship between the degree of expression of PD-L1 and CTLA-4 by stromal lymphocytes was demonstrated. A directly proportional correlation of PD-L1 expressing ST immune cells with ST CD4+ and FoxP3 lymphocytes was also found. One can assume the leading role of PD-L1 as an inhibitory mechanism of action in the stroma in these tumors, and FoxP3 T-regulatory cells (as a subtype of CD4+ T lymphocytes) are executors of the suppressive function.

The expression levels of PD-L1 and CTLA-4 in tumor and immune cells do not have sufficient prognostic value as independent biomarkers in the BC cases we studied. Despite the lack of significant association with survival, finding correlations with some of the classic prognostic factors will allow to clarify the role of these checkpoint molecules in our study.

It was found that PD-L1 positive stromal immune cells "preferred" mainly G3 carcinomas and only in them a high concentration was reported, and all heavily infiltrated neoplasms were of the TN subtype. Also, the tumor cells of the poorly differentiated carcinomas more often lacked positivity for CTLA-4, compared to the G1 and G2 neoplasms.

In the basal subtype of BC, a high concentration of ST PD-L1+ immune cells and expression (including high) of PD-L1 in the tumor cells is more often found, compared to the non-basal.

Some correlations were also found in the histological variants studied. In the category with another (special) morphological type of neoplasms, a strong degree of infiltration with PD-L1 positive stromal immune cells is more often reported than their lower values. In lobular carcinomas, however, no stromal PD-L1 positive cells were detected, but more often there was a high expression of CTLA-4 in the tumor cells. With NST subtype, no statistically significant difference was found for the studied checkpoint indicators.

The results indicated are consistent with some of those mentioned above, according to which TNBC is one of the predominant subtypes in the G3 neoplasm categories, with a basal phenotype and a special morphological type, the only one in our study with a high concentration of stromal PD-L1+ cells and with a high tumor expression for this molecule. In addition, HER2-positive carcinomas (with the same relative proportion) and, more rarely, LumB tumors, show a low degree of differentiation. Also, the basal phenotype can be found in all surrogate molecular subtypes of BC. In addition, tumors with other morphologic types were often included in the LumA variant group, but no case with stromal PD-L1 positive immune cells was found. From this, it can be hypothesized that PD-L1 positive immune cells in the stroma are involved in IR mostly in the poorly differentiated tumors, basal-like subtype and special histological type of each

surrogate molecular subtype other than the LumA variant. However, they are found in high concentration only when BC has a TN phenotype. In addition, it can be said that in these cases the inhibitory activity of the PD-L1 dependent mechanism is sufficient to suppress IR. Probably for this reason, no significant correlations were found for the CTLA-4 molecule, indicating its involvement in immune activity in neoplasms with a special (non-lobular) morphological type, basal subtype and with a low degree of differentiation.

In relation to lobular carcinomas, the inverse correlation was observed for the studied inhibitory molecules, with a more pronounced involvement of CTLA-4 positive tumor cells. In our study, the majority of neoplasms of this type were luminal subtypes (with a slightly higher relative proportion of the LumB variant). The results of the correlation analysis of the immune checkpoint molecules and the clinico-pathological data confirm the above described CTLA-4 inhibitory mechanism is mainly involved in the LumA variant. They also show that its activity can also be expected in the LumB subtype when the carcinoma is of the lobular type.

In the majority of investigated cases with metastatic LN, the absence of stromal CTLA-4 positive immune cells is more often found, and in non-metastatic LN – their high concentration. Also, cases with a high concentration of stromal CTLA-4 positive immune cells predominate in TNBC with LVI, and they are more often not observed in the absence of such invasion.

These correlations may be explained in part by the findings that lymphogenic spread of carcinomas is less likely for LumA and TN subtypes. It is with the LumA variant that the high concentration of stromal CTLA-4 positive immune cells is most often reported. On the other hand, the role of CTLA-4 in TNBC is secondary. We hypothesize that CTLA-4 stromal activity alone has a beneficial effect on the tumor microenvironment in LumA, improving the nodal status of patients. It is also possible that additional tumor and/or other dominant immune factors prevent the inhibitory effect of this checkpoint molecule from manifesting. In the TN subtype, however, the CTLA-4 mechanism likely contributes to its albeit less frequent lymphatic spread. It is possible that it affects the activity of immune cells, which would prevent contact between tumor and endothelial cells.

Conclusion:

In summary of the data from the literature review and the results of our study, it can be said that IR in BC is complex and not fully studied. The individual components of IS show a heterogeneous distribution in the tumor area (stromal and parenchymal) and have different involvement in BC subtypes (histological and molecular). It is proposed to apply a complex approach with consideration of several immune factors - %TIL, subtypes of immune cells - incl. B-, T- and T - subtypes of lymphocytes, as well as checkpoint molecules - incl. PD-L1 and CTLA4. Determining a basal phenotype in BC would add to the significance of

the results of the studied immunological components. Clinical validation and standardization of the approach may allow for more accurate prediction and personalized treatment in patients with BC.

VI. CONCLUSIONS

Conclusions related to the frequency and prognostic value of epidemiological and clinico-pathological characteristics of the studied cases with BC and their differences in surrogate molecular subtypes - LumA, LumB, HER2 and TN:

1. The frequency of BC increases after the age of 30 and reaches its peak in 70-79 year-old patients, mainly of female sex. In younger patients (<50 years of age) Luminal A subtype BC is mainly diagnosed, and in later age mainly the other variants (Luminal B, HER2 positive non-luminal and TN) are found.
2. The predominant histological type is NST, which is diagnosed most often in patients with the HER2 subtype. Lobular carcinoma is found in the smallest percentage of cases studied and is predominantly Luminal B subtype. Special types of BC are mainly in Luminal A and TN categories.
3. Special types with a favorable prognosis can be found both in Luminal A and in TNBC. TN subtype includes those with unfavorable prognosis.
4. Among the surrogate molecular subtypes, Luminal A is associated with the most favorable clinicopathological factors.

- Patients with this subtype are mostly diagnosed in the I^{-th} clinical stage, more often they have a small tumor size (<3 cm), which is highly differentiated - G1, in most cases without lymphovascular invasion (LVI) and without metastatic spread in axillary lymph nodes. The highest relative proportion of 5-year overall survival was also found in patients with this variant.

5. TN, HER2 and Luminal B subtypes are associated with adverse clinicopathological factors, heterogeneously presented in the different subtipes of BC.

- Advanced clinical stage (III^{-rd} and IV^{-th}), larger tumor size, presence of LVI and lymphogenous spread in the LN are found mostly in LumB subtypes and HER2 positive non-luminal BC. The high histological grade – G3, occurs mainly in the HER2 positive and TN subtype. The shortest overall survival is characteristic of patients with HER2 and TNBC.

6. A basal-like phenotype is most typical of TN neoplasms, mostly with a special variant, and is found less often in the other subtypes of BC.

- A basal-like phenotype is most often found in TNBC, mainly in other morphological types of neoplasms.

7. Basal-like features are associated with an unfavorable prognosis for patients.

- Patients with this type of tumor more often have <5-year overall survival.

Conclusions based on determining the frequency of immune factors in the studied cases with BC, their mutual correlation and their differences in Conclusions based on determining the frequency of immune factors in the studied cases with BC, their mutual correlation and their differences in surrogate molecular subtypes - LumA, LumB, HER2 and TN:

1. LPBC - with TILs >50%, is found in a small part of patients with this neoplasm, mainly with HER2 and TN subtypes.
2. CD3+ T-cell-mediated immunity is leading in the antitumor response in BC, and humoral CD20+B-cell is less widespread.
3. Cytotoxic CD8+ T-lymphocytes are the predominant T-cell subtype, both stromally and intratumorally.
4. CD8-Tc and FoxP3-Treg subtypes of TILs are more often found together intratumorally and stromally, mainly in the TN subtype.
5. The stroma is a preferred site for infiltration by most types of lymphocytes (CD20+ B, CD3+ T, CD8+ cytotoxic and FoxP3+ regulatory), regardless of their concentration and BC subtype in which they settle.

- Exceptions are high-concentration CD4+ T-helpers, which are more common intratumorally in HER2 and TN variants of breast cancer.

6. Intratumoral and stromal, low concentrations of lymphocyte subtypes are more often found. A high concentration is less common, being characterized primarily by CD3 T-lymphocytes, mainly the CD8+ cytotoxic and FoxP3+ regulatory subtype.
7. TN and HER2 positive BC are more immunogenic subtypes compared to luminal, but a subpopulation is also found in the LumB variant, with pronounced antitumor IR.

- The high degree of infiltration of intratumor lymphocyte subtypes is most characteristic for and HER2 subtypes. This corelation was also found for stromal localization of CD20, CD8 and FoxP3 cells.
- The high stromal concentration of CD3 and CD4, in addition to TN and HER2, is also characteristic of the Luminal B subtype.

8. Inhibitory mechanisms have a different role in the molecular subtypes of BC, as TN (in which the basal-like subtype of neoplasms are most often found) is a preferred subtype for the inclusion of the checkpoint suppressor pathways - with the participation of PD-L1 in a major role, a secondary – of the CTLA-4 molecule.

- In TNBC, intratumoral and stromal PD-L1 positive immune cells are most often found;
- TN is the BC subtype in which tumor expression for PD-L1 is most commonly reported;
- CTLA-4 positive intratumoral immune cells are found only in TNBC.

9. Luminal A is a preferred subtype of BC for involvement of the CTLA-4 checkpoint mechanism.

- CTLA-4 positivity in tumor and stromal immune cells, incl. with their high concentration, is reported most often in LumA, compared to the other subtypes of BC.

Conclusions related to correlations between epidemiological and clinicopathological data with immune response factors, incl. with prognostic value

1. High TILs concentration is associated with unfavorable epidemiological and clinicopathological prognostic factors.

- TILs >50% is found in poorly differentiated carcinomas - G3 and mainly in patients with metastatic LN.
- High TILs concentration is associated with <5 year overall survival in patients.

2. In BC, intratumoral FoxP3 T-regulatory cells, regardless of their quantity, are an unfavorable prognostic factor when combined with a low concentration of intratumoral CD4 helper T-lymphocytes.

- In G3 cancers, a CD3 T-cell IR associated with the participation of mainly intratumoral FoxP3 regulatory T-lymphocytes (regardless of their concentration) and a weak presence of intratumoral CD4 helper T-cells was found.

3. A high concentraion of regulatory FoxP3+ T - cells in the stroma is associated with a negative prognosis.

- A high concentration of ST FoxP3 cells is more often found in G3 carcinomas compared to more highly differentiated carcinomas.

4. More efficient cytotoxic activity is suggested in the special types of BC.

- In the category of other morphological (special) type of BC, a high concentration of intratumoral CD8+ and absence of IT FoxP3 were more often reported, compared to NST and lobular histological variant.

5. The presence of intratumoral CD20 and the high concentration of CD8 in the stroma of HER2, as well as the high concentration of stromal CD20 in TN subtype carcinomas act as an unfavorable prognostic factor in the corresponding subtypes of neoplasms.

- LVI is associated with high concentration of ST CD8 in HER2 positive carcinomas and with high concentration of ST CD20 in TN subtype.
- Metastatic spread in LN in TNBC is also associated with a high concentration of ST CD20.
- Longer (>5 years overall) survival in patients with HER2 subtype was associated with absence of IT CD20, and shorter survival was found to be low (considering that a high concentration of this type of cells was not reported in none of the studied cases).

6. The presence of CD8+ T-lymphocytes with intratumoral localization is associated with a favorable prognosis in LumA tumors.

- Tumors ≤3 cm in size in the LumA subtype mostly have a low concentration of IT CD8, and in larger tumors such cells are absent (and their high concentration is not observed at all in this subtype of BC).

7. The reporting of the lymphocyte infiltrate in a larger surgical specimen will allow a more precise determination of IR in BC.

- In mastectomy, a higher concentration of ST CD3, IT CD3 and IT CD4 is more often reported than in excisional type biopsy, where their low concentration prevails.

8. Checkpoint molecules show varied involvement in different histological variants of BC.

- CTLA-4 is found mainly in lobular BC, and PD-L1 in other special types.

9. PD-L1 is a major inhibitory mechanism in the stroma of Luminal B subtype BC, and FoxP3 T-regulatory cells (as a subtype of CD4+ T lymphocytes) perform the suppressor function.

- There is a linear correlation for PD-L1, CD4 and FoxP3 positive stromal cells in LumB BC.

10. The involvement of PD-L1 related inhibitory mechanism is more typical of basal subtype BC, compared to non-basal.

- In a basal-like phenotype, expression (incl. high) for PD-L1 on tumor cells and a high concentration of ST PD-L1+ immune cells are more often found, compared to the non-basal type BC.

11. Of the luminal subtypes of BC, the CTLA-4 inhibitory mechanism is mainly involved in the LumA variant, and its activity can be expected in the LumB subtype, especially when the carcinoma is of the lobular type.

- In lobular subtype of BC, CTLA-4 expression is more often found in the tumor cells.

- Most of the lobular carcinomas are luminal variants, with a greater relative proportion for LumB.

12. The CTLA-4 checkpoint molecule probably probably has a suppressive effect in HER2 subtype BC on the infiltration of CD4+ T-helpers and the activity of cytotoxic T-lymphocytes in the tumor area.

- CTLA-4 expression on tumor and stromal immune cells correlates inversely with the intratumoral concentration of helper CD4+ T cells in this subtype of BC.

- There is a unidirectional correlation between tumor positivity for CTLA-4 and stromal concentration of cytotoxic CD8+ T cells.

13. The high concentration of PD-L1+ stromal immune cells is found only in G3 carcinomas when the neoplasms are TN subtype and is more common in those with special type and BC with basal phenotype (with molecular subtype other than LumA).

- PD-L1 positive cells in the stroma are part of the immune response mostly in G3 carcinomas, compared to G1 and G2 neoplasms, and their high concentration is found only in poorly differentiated tumors, all of which are TN subtype.

- PD-L1 stromal cells are not part of IR in LumA subtype.

- In a special histological type and in basal-like carcinomas, a high concentration of PD-L1 stromal cells is more common than a low.

14. Lack of positivity for CTLA-4 in tumor cells and high concentration of stromal PD-L1+ immune cells are associated with one of the classic unfavorable prognostic factors – low degree of differentiation of neoplasms - G3.
15. It is assumed that patients (mostly with TNBC) with high PD-L1 expression on tumor and immune cells, in combination with high intratumoral concentration of CD8+, FoxP3 and CD20+, are most suitable for immune therapeutic modulation.
16. Prognosis in BC subtypes is a complex indicator.

- Classical prognostic factors are not unambiguous and definitive enough.
- It is necessary to consider more than one clinical-pathological factor, incl. finding new prognostic biomarkers

VII. CONTRIBUTIONS OF THE DISSERTATION

Contributions of a scientific-practical and/or original nature

1. For the first time in Bulgaria, IR factors were studied in all surrogate molecular subtypes of BC.
2. For the first time in the country, computer-assisted morphometric analysis of lymphocyte subtypes - B-, T- and subtypes T - helper, cytotoxic and regulatory were performed.
3. For the first time in Bulgaria and abroad, a complex assessment of immune factors was made for individual surrogate molecular subtypes of BC: TILs, major subtypes of lymphocytes and main inhibitory immune "checkpoint" molecules, in addition to determining an aggressive basal phenotype in neoplasms.
4. A methodology for determining TIL subtypes, basal phenotype and some checkpoint molecules in BC in pathological practice is applied - **Appendix 1, 2 and 3**.

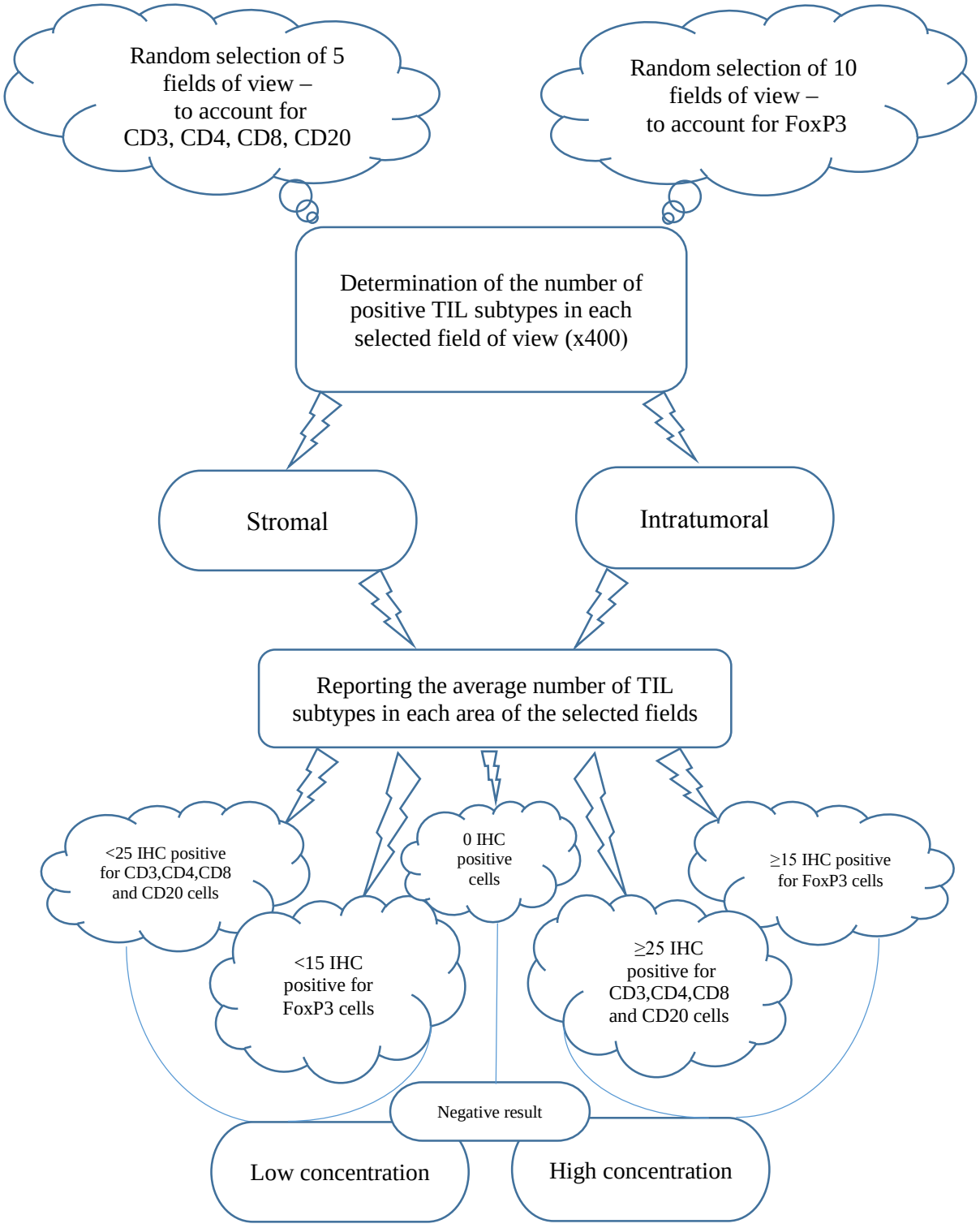
Contributions of a scientific and theoretical nature

1. Immunophenotyping of T-cell subpopulations and immune “checkpoint” molecules PD-L1 and CTLA4, as well as determination of basal subtype can be discussed in the context of their use as a prognostic marker in patients with BC.
2. A complex approach is proposed for immunohistochemical determination of prognostically significant factors in different surrogate molecular subtypes of BC - **Appendix 4**.
3. The obtained results can be used for subsequent clinical validation of the prognostic value of immune factors in a larger number of patients.
4. The established dependencies may serve as a database for additional research, with the aim of incorporating them into a prognostic algorithm and predictive morphological screening, allowing a better selection of patients for subsequent, chemo-, immuno-, targeted or other type of therapy in patients with BC.

VIII. APPLICATIONS

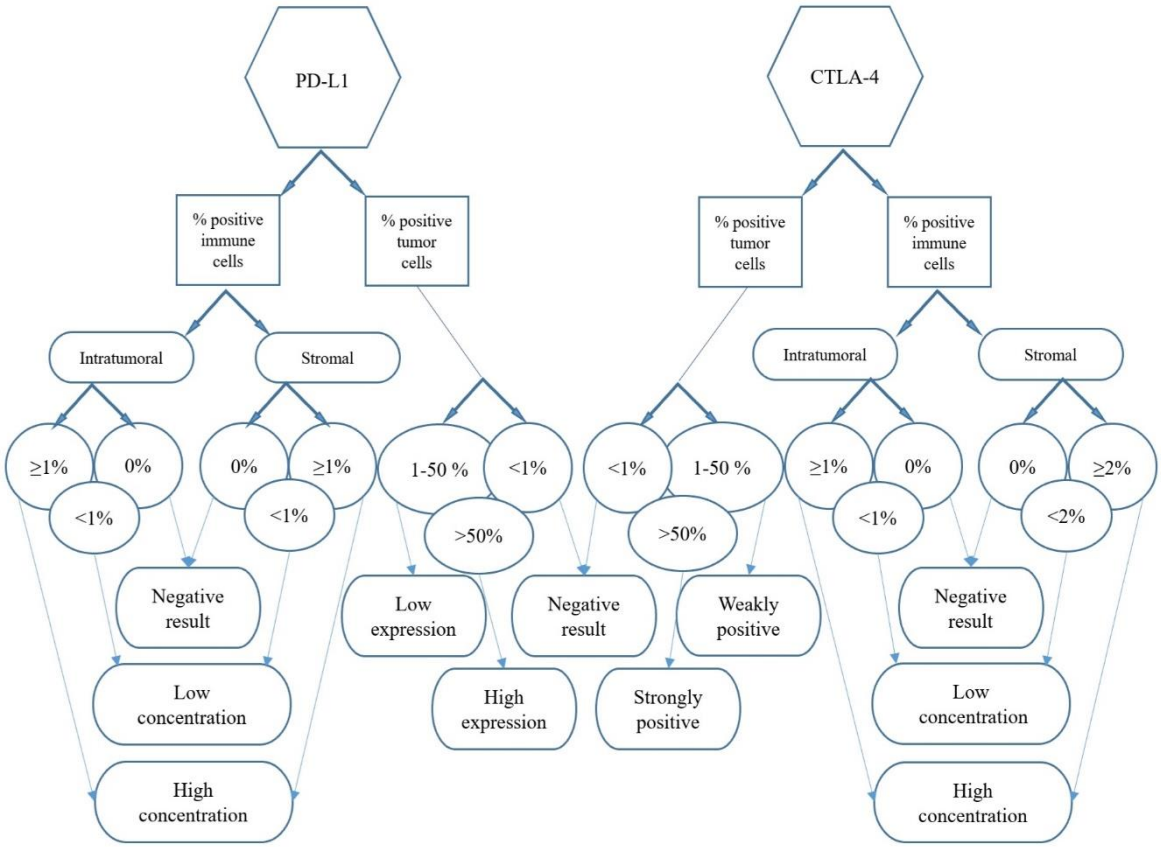
Application 1

Methodology for microscopic assessment of TIL subtypes - CD3, CD4, CD8, CD20 and FoxP3 lymphocytes in BC



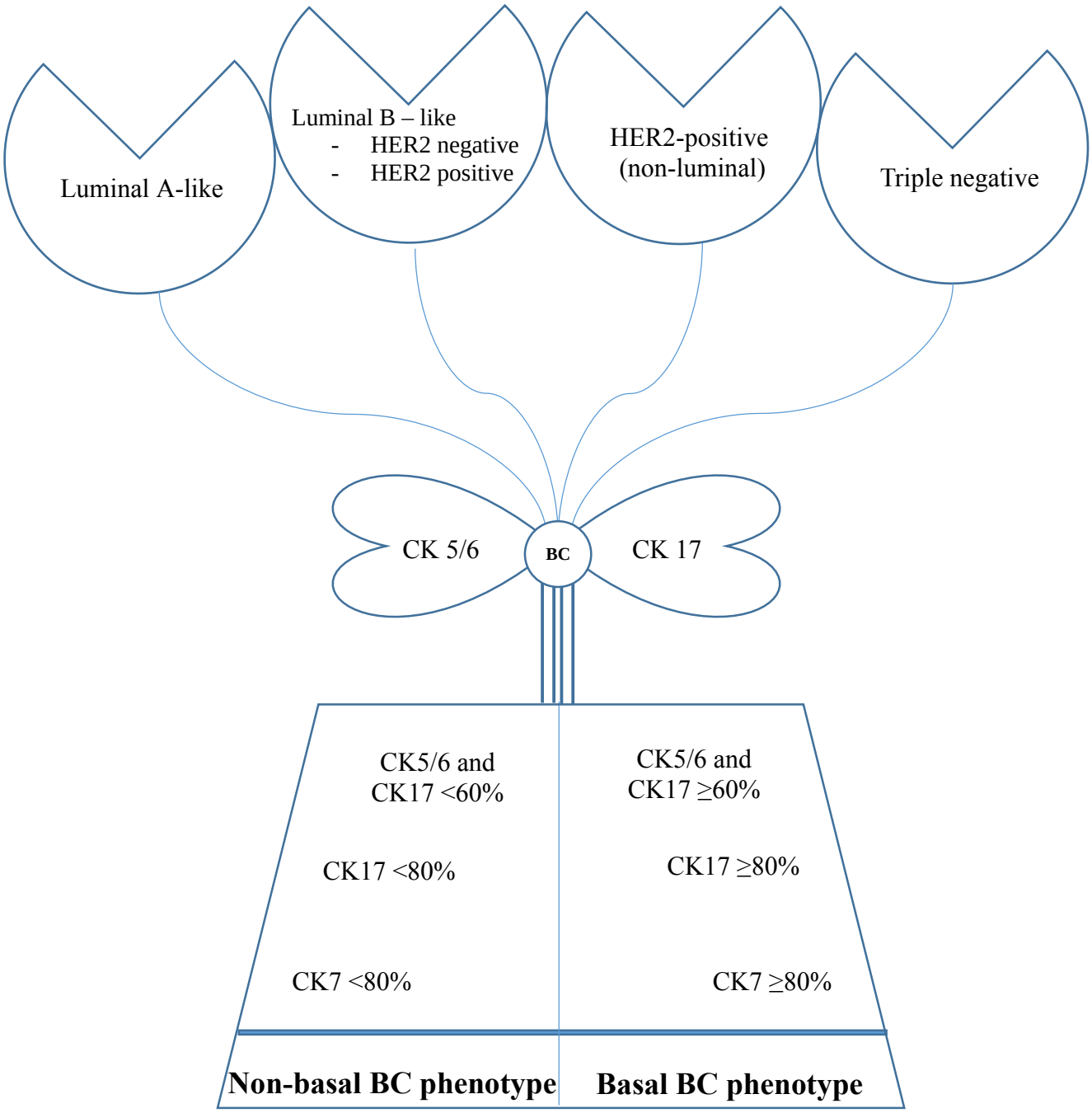
Application 2

Methodology for microscopic assessment of PD-L1 and CTLA4 in tumor and immune cells in BC



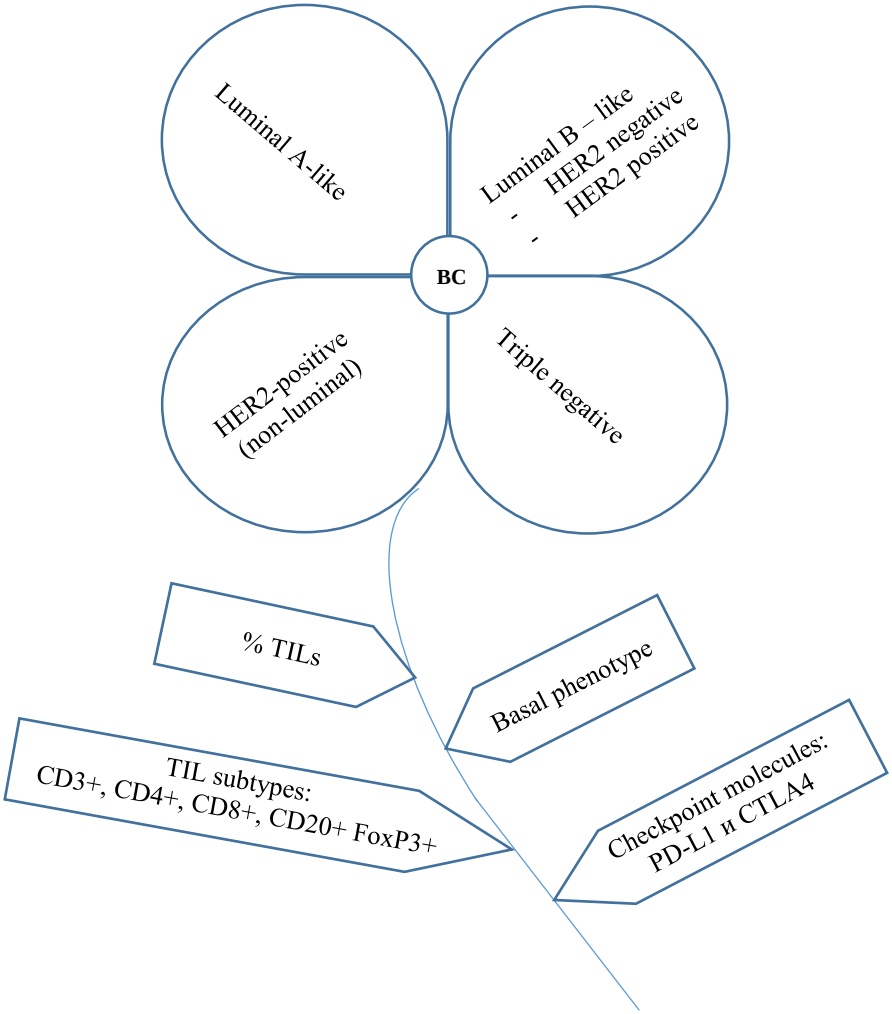
Application 3

Methodology for immunohistochemical determination of basal phenotype in BC



Application 4

Complex approach for immunohistochemical determination of prognostically significant factors in different surrogate molecular subtypes of BC



IX. PUBLICATIONS, PARTICIPATION IN SCIENTIFIC FORUMS, COURSES AND PROJECTS RELATED TO THE DISSERTATION

1) **Publications** of scientific results in **full-text articles** and/or reports **related to the dissertation work:**

1. Polina D. Dimitrova, Savelina L. Popovska, Ivanov N. Ivanov. „A study on tumor-infiltrating lymphocytes in different subtypes breast cancer“, J Biomed Clin Res, Vol.14, No1, 2021, p.70-81 (ISSN 1313-6917), Online (ISSN 1313-9053)
2. Dimitrova PD, Popovska SL, Ivanov IN, Dineva TB. Expression of Immune Checkpoint Molecules - PD-L1 and CTLA-4 in Different Subtypes of Breast Cancer. Akusherstvo i Ginekologiya 2019, 58(3): 23-29, (SJR= 0,12); ISSN 0324-0959

3. Popovska S., Damianova P., Tomov S., Dineva T., Ivanov I. Case of encapsulated solid papillary carcinoma with triple-negative and basal-like phenotype occured in pregnant woman with review of the literature. Akusherstvo i Ginekologiya 2015, 54 (2): 50 – 56. ISSN 0324-0959 (IF =0.14)

2) Participation in scientific forums related to the dissertation work:
- in the country

1. 17-18.06.2022 - National Conference on Pathology, Lukovit, Bulgaria
   - S. Popovska, P. Damyanova, A. Yordanov, M. Vasileva – Slaveva. Role of CD47 in cervical cancer. Collection of abstracts, page 17.; National Pathology Conference; 17-18.06.2022; SSR
2. 03-04.06.2022 - Academy of Molecular Pathology - Digital Breast Pathology, Pleven, Bulgaria
   - P. Damyanova. Macroscopic processing of breast samples according to recommendations. Presentation.
3. 28-30.01.2022 – II^{-nd} scientific-practical conference Cardio-oncology - team care for better results, interdisciplinary approach for different nosologies.
   - P. Damyanova. Diagnosis of breast cancer – the pathologist's point of view. Presentation.
4. 10-12.09.2021 – XIII National Congress of Pathology - Burgas, Bulgaria
   - P. Damyanova, S. Popovska. Immunohistochemical expression of BRCA1 associated protein in basal-like subtype of breast cancer. Collection of abstracts XIII National Congress of Pathology - 10-12.09.2021, Burgas; EP-8, 52-53 p

- P. Damyanova. Triple negative breast carcinoma - role of precise pathology - morphology and IHC subtypes. Molecular subtyping. Predictive biomarkers with a focus on PD-L1.

5. 31.10-02.11.2019г - Jubilee Scientific Conference, MU-Pleven, Pleven, Bulgaria

- Polina D. Dimitrova, Savelina L. Popovska, Ivan I. Ivanov, Tereza B. Dineva. Study on the tumor immune microenvironment in different subtypes of breast cancer. Poster - PSO15.

6. 11.04. – 14.04.2019 - National Conference of Pathology, Velingrad, Bulgaria

- P. Damyanova, S. Popovska, I. Ivanov, T. Dineva. Study on the expression of PD - L1 and CTLA - 4 in the different molecular subtypes of breast cancer, National Conference on Pathology, 11-14.04.2019, Velingrad - poster, Collection of Summaries, 83-84 p

7. 11-13.05.2017 – XII National Congress of Pathology, Veliko Tarnovo, Bulgaria

- S. Popovska, P. Damyanova, I. Ivanov, K. Petrov, R. Ivanova, T. Dineva. Immunity and breast cancer. XII National Congress of Pathology - V. Tarnovo, presentation; 35-36 p.

8. 17-19.06.2016 – Fourth annual scientific conference with international participation of Bulgarian Association of Medical Oncology (BAMO) , Sofia

- S. Popovska, P. Damyanova, I. Ivanov, T. Dineva. Study on distribution, localization and immunophenotype of tumor infiltrating lymphocytes in different molecular subtypes of breast cancer, presentation

- abroad

1. 29-31.08.2021 – 33-rd European congress of Pathology -Belgium (Virtual (Virtual Congress)

- P. Dimitrova, S. Popovska, I. Ivanov. A study on tumour-infiltrating lymphocytes, PD-L1 and BRCA1 immunohistochemical expression in basal-like subtype of breast cancer. Virchows Archiv (2021) 479 (Suppl 1):S1–S320; PS-02-009, S68; https://doi.org/10.1007/s00428-021-03157-8, ISSN 0945-6317, ISSN (print), 1432-2307 (web), (IF=3.31, 2020).

2. 05-09.12.2017 – San Antonio breast cancer symposium, San Antonio, Texas, USA

- Popovska S., Dimitrova P., Dineva T. A study on amount, localization and immune phenotype of tumor-infiltrating lymphocytes in different subtypes of breast cancer. CANCER RESEARCH, Vol: 78, Iss: 4 , Suppl: S , Meeting Abstract: P3-05-20, FEB 2018, (IF 2018 – 8.37) DOI: https://doi.org/10.1158/1538-7445.SABCS17-P3-05-20

3) Participation in specialized courses in the field of the topic of the dissertation work:

1. 02-04.05.2019 - EScoP Varna: Update in Breast Pathology, Varna, Bulgaria
2. 10.12.2020; 04.03.2021 - PD-L1 (SP142) TNBC Training, Roche, Bulgaria
3. 01-05.07.2019 - „Erasmus+“, Section of Anatomic Pathology, Bellaria Hospital, Department of Biomedical and Neuromotor Sciences Bologna, Italy
4. 19-20.09.2018 – Breast pathology masterclass, pathology masterclass, Rome, Italy
5. 18-20.10.2017 – 4-th annual the course – Diagnostic Immunohistochemistry for Pathologists, Pathologists, Krakow, Poland
6. 25-26.09.2017 – HER2 interpretation training in breast and gastric carcinoma, Sofia, Bulgaria

4) Participation in scientific projects

- **directly related** to the dissertation:

1. Study on the expression of BRCA1 and BRCA2 related proteins in different molecular subtypes of breast cancer determined by immunohistochemical method (RESEARCH PROJECT No. D2, funding year 2020, MU - Pleven)
2. Study on the expression of the immune checkpoint inhibitors PD - L1 and CTLA - 4 in the different molecular subtypes of breast cancer (RESEARCH PROJECT No. 11, funding year 2018, MU- Pleven)
3. Study on distribution, localization and immunophenotype of tumor infiltrating lymphocytes in different molecular subtypes of breast cancer (RESEARCH PROJECT No. 19, funding year 2015, MU-Pleven)
4. European Regional Development Fund through the: Operational Programme "Science and Education for Smart Growth", with a leading organization MU-Pleven, grant no BG05M2OP001-1.002-0010-C01 (2018-2023)