Uniwersytet Jagielloński
Collegium Medicum
Wydział Lekarski

mgr Anna Glajcar

Ocena mikrośrodowiska nowotworowego w różnych podtypach molekularnych raka piersi

The evaluation of tumor microenvironment in distinct breast cancer molecular subtypes

Praca doktorska

Promotor: dr hab. n. med. Krzysztof Okoń, prof. UJ
Promotor pomocniczy: dr n. med. Joanna Szpor

Pracę wykonano w Katedrze Patomorfologii UJ CM
Kierownik jednostki: prof. dr hab. n. med. Dariusz Adamek

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Anna Glajcar

Spis treści

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1. **Anna Glajcar**, Joanna Szpor, Agnieszka Pacek, Katarzyna Ewa Tyrak, Florence Chan, Joanna Streb, Diana Hodorowicz-Zaniewska, Krzysztof Okoń: The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment.
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2. **Anna Glajcar**, Joanna Szpor, Katarzyna Ewa Tyrak, Joanna Streb, Diana Hodorowicz-Zaniewska, Krzysztof Okoń: Lymphoid environment in molecular subtypes of breast cancer.
   Pol J Pathol, 2018, 69 (2): 169-181.

3. **Anna Glajcar**, Joanna Szpor, Diana Hodorowicz-Zaniewska, Katarzyna Ewa Tyrak, Krzysztof Okoń: The composition of T-cell infiltrate varies in primary invasive breast cancer of different molecular subtypes as well as according to tumor size and nodal status.
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Wstęp

Mikrośrodowisko nowotworowe stanowią elementy bezpośrednio otaczające guz nowotworowy, takie jak: fibroblasty, macierz komórkowa oraz komórki stanu zapalnego. Różnorodność populacji komórek odpowiedzi immunologicznej oraz substancji przez nie wydzielanych przekłada się na ich zdolność do niszczenia komórek nowotworowych lub - przeciwnie - wywoływania zjawiska tolerancji immunologicznej. Uważa się, że wpływ na przebieg choroby nowotworowej mają zarówno komórki odpowiedzi swoistej – limfocyty B i T (oraz ich subpopulacje – limfocyty regulatorowe, cytotoksyczne i pomocnicze) – jak i komórki odpowiedzi nieswoistej, takie jak mastocyty czy komórki NK. Jednocześnie substancje wydzielane przez komórki guza nowotworowego mogą wpływać na aktywność, fenotyp, oraz rekrutację do mikrośrodowiska poszczególnych populacji komórek zapalnych. W rezultacie skład ilościowy oraz jakościowy mikrośrodowiska nowotworowego może zarówno hamować rozwój choroby nowotworowej, jak i przyczyniać się do jej progresji.

Obecnie inwazyjny rak piersi postrzegany jest jako heterogenna grupa guzów, które wywodzą się z nowotworowo przekształconych komórek nabłonkowych wyścielających końcowe fragmenty gruczołu sutkowego (przewodzików i zrazików). Poszczególne podtypy raka piersi różnią się wzorami mutacji genetycznych, a zmiany te mają swoje odzwierciedlenie w różnych fenotypach złośliwych guzów piersi. Zgodnie z zaleceniami z St. Gallen, podtypy molekularne określa się na podstawie ekspresji czterech markerów białkowych: receptora estrogenowego (ER), progesteronowego (PR), oraz receptora naskórkowego czynnika wzrostu typu 2 (HER2), a także białka Ki67, które jest markerem intensywności proliferacji komórek nowotworowych. Poszczególne fenotypy złośliwych guzów sutka charakteryzują się różną agresywnością kliniczną - raki piersi wykazujące ekspresję ER i/lub PR (luminalne) cechują się wolniejszym tempem wzrostu i mniejszą skłonnością do nawrotów i tworzenia przerzutów, podczas gdy nadekspresja HER2, wyższa ekspresja Ki67, brak ekspresji ER/PR (podtyp nieluminalny), a także fenotyp ER/PR/HER2-ujemny (*triple-negative breast cancer*, TNBC) stanowią niekorzystne czynniki rokownicze dla przebiegu choroby.

Dotychczas zaobserwowano, że podtypy molekularne raka piersi różnią między sobą gęstością nacieku jednojądrzastych komórek zapalnych. Jednocześnie zależności pomiędzy występowaniem poszczególnych populacji komórek zapalnych w mikrośrodowisku nowotworowym a podtypem molekularnym inwazyjnego raka piersi nie zostały jednoznacznie określone.

Cel pracy

Celem niniejszej pracy było określenie różnic w gęstości nacieku wybranych populacji komórek stanu zapalnego (mastocytów chymazo- i tryptazo-dodatnich, limfocytów T i B, komórek NK, komórek cytotoksycznych, regulatorowych i limfocytów pomocniczych Th2) w pierwotnych inwazyjnych guzach piersi w zależności od:

- podtypu molekularnego inwazyjnego raka piersi (określonego na podstawie klasyfikacji z St. Gallen);
- ekspresji markerów białkowych o znaczeniu prognostycznym i predykcyjnym w raku piersi, takich jak ER, PR, HER2, Ki67;
- występowania innych czynników rokowniczych w raku piersi (wielkość guza, stopień zajęcia węzłów chłonnych, stopień złośliwości histologicznej, podtyp histologiczny guza).

Materiał i metody

Badania przeprowadzono na archiwalnym materiale tkankowym przechowywanym w Katedrze Patomorfologii Collegium Medicum Uniwersytetu Jagiellońskiego. W pierwszej i drugiej pracy materiał stanowiło 108, zaś w trzeciej pracy 106 wycinków tkankowych zdiagnozowanych jako pierwotny inwazyjny rak piersi. Kryterium wykluczenia stanowiło przedoperacyjne zastosowanie leczenia systemowego. Podtyp molekularny raka piersi ustalono w oparciu o klasyfikację z St. Gallen (w pierwszej pracy przyjęto klasyfikację z 2013 roku, w dwóch kolejnych pracach zastosowano klasyfikację z 2015 roku). Po standardowym utrwaleniu i zatopieniu materiału tkankowego, z bloczków parafinowych przygotowane zostały skrawki o grubości 4 μm, które następnie zostały wybarwione immunohistochemicznie w kierunku ekspresji chymazy, tryptazy, CD45RO, CD20, CD56, CD8, FOXP3 oraz GATA3 celem identyfikacji analizowanych populacji komórek nacieku zapalnego. Odczyny immunohistochemiczne wykonano metodą manualną, stosowaną rutynowo w Katedrze Patomorfologii. Gęstość nacieku pozytywnie wybarwionych komórek oceniono w mikroskopie świetlnym. Liczebność mastocytów, komórek NK, cytotoksycznych i regulatorowych wyrażono jako sumę liczb pozytywnie wybarwionych komórek uzyskanych z 5 pól widzenia o powiększeniu 400x (co odpowiadało 1 mm^2 tkanki), zarówno w obrębie nowotworowo zmienionego nabłonka jak i w bezpośrednio otaczającej go tkance. W tych samych lokalizacjach oceniono odsetek tkanki zajętej przez limfocyty T i B, na podstawie średniej z 5 pól widzenia o powiększeniu 100x. Dodatkowo, dla komórek cytotoksycznych, regulatorowych i Th2 oszacowano ich procent w otaczającym guz nacieku komórek jednojądrzastych, na

podstawie średniej wartości otrzymanej z 5 pól widzenia o powiększeniu 400x. Otrzymane wyniki odniesiono do danych patologiczno-klinicznych, a całość poddano analizie statystycznej przy użyciu programu Statistica.

Wyniki i wnioski

Zaobserwowano różnice w gęstości nacieku analizowanych komórek w zależności od podtypu molekularnego raka piersi. Dla mastocytów tryptazo- i chymazo-dodatnich analiza post-hoc wykazała istotnie gęstszy naciek tych komórek zlokalizowanych wewnątrz guzów o fenotypie luminalnym A i B, w porównaniu z nowotworami HER2-dodatnimi nieluminalnymi potrójnie ujemnymi (TNBC). Odwrotne zależności zaobserwowano dla komórek limfoidalnych; spośród wszystkich podtypów nowotwory luminalne A charakteryzowały się naciekiem limfocytów T o najmniejszej intensywności, zarówno wewnątrz guza (istotna różnica w porównaniu z nowotworami TNBC) jak i na jego krawędzi (istotne różnice wykazano w porównaniu z guzami TNBC, HER2-dodatnimi nieluminalnymi i luminalnymi B z nadekspresją HER2). Analiza post-hoc wykazała także istotnie mniej gęsty naciek limfocytów B występujących na krawędzi zmian luminalnych A w porównaniu do raków TNBC i HER2+ nieluminalnych. Podobnie, fenotyp luminalny A oraz B był związany z mniejszą gęstością komórek NK zlokalizowanych na pograniczu guza w porównaniu z nowotworami TNBC. Obserwowana liczebność komórek cytotoksycznych i regulatorowych na krawędzi guza była istotnie wyższa w podtypach HER2-dodatnich nieluminalnych i TNBC niż w guzach luminalnych A; druga z poddanych zależności dla komórek regulatorowych była obserwowana także wewnątrz zmiany nowotworowej. Dodatkowo, gęstość nacieku komórek regulatorowych na krawędzi zmiany różniła się pomiędzy podtypami luminalnymi A i B, z ich większą liczebnością w zmianach drugiego typu. Większą wartość proporcji procentowych udziałów w nacieku komórek regulatorowych/Th2 zaobserwowano w guzach HER2-dodatnich nieluminalnych w porównaniu z guzami luminalnymi A.

Zależności pomiędzy zwiększoną gęstością nacieku a występowaniem nadekspresji HER2 zaobserwowano wyłącznie na krawędzi guza dla limfocytów T, limfocytów B, komórek regulatorowych i cytotoksycznych. Odwrotną zależność, również dookoła zmiany nowotworowej, wykazano dla mastocytów chymazo- i tryptazo-dodatnich.

Wykazano dodatnią korelację pomiędzy ekspresją ER i PR a liczebnością mastocytów chymazo- i tryptazo-dodatnich, oraz odsetkiem w nacieku komórek Th2 i stosunkiem odsetków komórek cytotoksycznych/Th2; ujemną korelacje wykazano dla gęstości nacieku limfocytów T, limfocytów B, komórek NK, komórek cytotoksycznych, komórek regulatorowych (zarówno

dla liczebności jak i odsetka komórek w nacieku) i stosunku procentowego udziału komórek regulatorowych/Th2. W odniesieniu do indeksu proliferacyjnego guza, zaobserwowano odwrotną zależność pomiędzy ekspresją białka Ki67 w komórkach nowotworowych a liczebnością mastocytów chymazo- i tryptazo-dodatnich, zaś dodatnią korelację zaobserwowano dla gęstości nacieku limfocytów T i B, komórek NK, komórek cytotoksycznych, regulatorowych (zarówno w liczbie jak i w odsetku komórek w nacieku) oraz stosunku procentowego udziału komórek regulatorowych/Th2.

Zaobserwowano zależność pomiędzy wyższym stopniem złośliwości histologicznej raka piersi a mniejszą liczebnością mastocytów tryptazo-dodatnich i zlokalizowanych wewnątrz guza mastocytów chymazo-dodatnich, a także bardziej intensywnym naciekiem limfocytów T i B, większą liczebnością komórek NK na krawędzi guza, większą liczebnością komórek cytotoksycznych i regulatorowych, zwiększonym odsetkiem komórek regulatorowych w otaczającym guz nacieku i wyższym stosunkiem odsetków komórek regulatorowych/ Th2.

Inwazyjne guzy piersi o średnicy większej niż 2 cm (pT>1) charakteryzowały się mniejszą liczebnością mastocytów tryptazo-dodatnich, a także gęstszym naciekiem limfocytów T, zlokalizowanych wewnątrz zmiany nowotworowej komórek cytotoksycznych i wyższym stosunkiem procentowego udziału komórek regulatorowych/Th2 w otaczającym guz nacieku zapalnym. Dodatkowo, w guzach luminalnych, wykazano zależność pomiędzy większym rozmiarem guza a gęstszym naciekiem komórek cytotoksycznych i regulatorowych wewnątrz guza, zwiększonym odsetkiem komórek regulatorowych w otaczającym nacieku oraz zwiększonym stosunkiem odsetków komórek regulatorowych/ Th2.

Wykazano zależność pomiędzy występowaniem przerzutów raka piersi w węzłach chłonnych a zwiększonym odsetkiem komórek regulatorowych oraz stosunkiem odsetków komórek regulatorowych/ Th2 w otaczającym guz podścielisku. W grupie raków nieluminalnych zaobserwowano wyższą wartość wskaźnika liczby komórek cytotoksycznych/ regulatorowych wewnątrz przerzutujących guzów.

W odniesieniu do typu histologicznego raka piersi, zmiany zakwalifikowane jako *no-otherwise specified* (NOS) cechowały się mniejszą liczebnością mastocytów tryptazo-dodatnich w obrębie nowotworowo zmienionego nabłonka, bardziej intensywnym naciekiem limfocytów T, limfocytów B, komórek cytotoksycznych i regulatorowych dookoła zmiany (zarówno pod względem ich liczby jak i odsetka w nacieku zapalnym), oraz zmniejszonym stosunkiem liczby komórek cytotoksycznych/ regulatorowych wewnątrz guza w porównaniu do guzów o morfologii zrazikowej.

Uzyskane wyniki wskazują na istnienie różnic w składzie mikrośrodowiska nowotworowego w zależności od podtypu molekularnego inwazyjnego raka piersi oraz występowania pozostałych czynników prognostycznych i predykcyjnych w tej chorobie.

- Różnice w nacieku poszczególnych komórek zapalnych oraz korelacje z ekspresją ER, PR i Ki67 wskazują na związek mastocytów z występowaniem podtypów raka piersi o łagodniejszym przebiegu klinicznym, podczas gdy bardziej obfity naciek komórek limfoidalnych i zwiększony stosunek odsetków komórek regulatorowych/ Th2 związane są z występowaniem podtypów cechujących się agresywnym przebiegiem choroby;
- Różnice w nacieku analizowanych komórek wskazują na związek mastocytów z występowaniem korzystnych czynników rokowniczych, takie jak mniejszy rozmiar guza, niższy stopień złośliwości histologicznej; odwrotna zależność obserwowana jest dla nacieku komórek limfoidalnych, cytotoksycznych i regulatorowych;
- Odmienne zależności obserwowane dla populacji komórek zapalnych zlokalizowanych wewnątrz guza lub na jego krawędzi wskazują na odmienną rolę tych komórek w patogenezie raka piersi w zależności od lokalizacji w tkance;
- Różnice w gęstości poszczególnych subpopulacji limfocytów T obserwowane pomiędzy luminalnymi i nieluminalnymi inwazyjnymi guzami piersi o różnym stopniu zaawansowania klinicznego choroby (rozmiar guza, zajęcie węzłów chłonnych) wskazują na odmienną rolę komórek stanu zapalnego w procesie progresji nowotworowej, w zależności od podtypu molekularnego raka piersi.

Introduction

Tumor microenvironment is made up of elements located in an immediate surrounding of a malignant tumor, such as: fibroblasts, stroma and cells of the immune system. The diversity of the immune cell populations and their mediators manifests in immune cell ability to kill cancer cells or, conversely, contributes to immunotolerance. It is accepted that cells of both the adaptive response – B-lymphocytes, T-cells (as well as their subpopulations – regulatory, cytotoxic and helper cells) - and the innate response (e.g. mast cells and NK cells) affect the course of cancer disease. In parallel, molecules secreted by cancer cells may impact on activity and phenotype of respective immune cell populations, as well as on their recruitment to the microenvironment. Consequently, composition of tumor microenvironment (in its quantitative and qualitative terms) is presumed to either inhibit or promote tumor progression.

The term 'invasive breast cancer' is currently related to heterogenous group of tumors, which derive from transformed epithelial cells that line terminal parts of mammary gland (ducts and lobules). Respective types of breast cancer tumors differ in their mutational pattern, which are reflected in a diversity of phenotypes observed in invasive breast cancers. In accordance with St Gallen recommendations, molecular subtypes are determined by expression of four protein markers: estrogen receptor (ER), progesterone receptor (PR) and human epithelial growth factor receptor 2 (HER2), as well as Ki67 protein, which indicates intensiveness of cancer cell proliferation. Respective phenotypes of invasive breast lesions are characterized by distinct clinical aggressiveness – breast tumors that express ER and/or PR (luminal tumors) show lower growth rate and lower metastatic potential, while overexpression of HER2, higher expression of Ki67, ER/PR-negativity (non-luminal type) and ER/PR/HER2-negative phenotype (triple-negative breast cancer, TNBC) are adverse prognostic factors in breast cancer.

To date, it has been observed that breast cancer molecular subtypes differ in a density of mononuclear cell infiltrate. However, relationships between the infiltrate of immune cell populations in tumor microenvironment and molecular subtypes of invasive breast cancer have not been fully elucidated yet.

Objectives

This study aimed to determine the differences in densities of selected immune cell populations (chymase- and tryptase-positive mast cells, T lymphocytes, B lymphocytes, NK cells, cytotoxic cells, regulatory cells and T-helper 2 cells) in primary invasive breast cancers, with regard to:

- a molecular subtype of invasive breast cancer (identified in accordance with St Gallen classification);
- an expression of protein markers with prognostic and predictive significance in breast cancer, such as ER, PR, HER2 and Ki67;
- the occurrence of other prognostic indicators in breast cancer (tumor size, lymph node involvement, histologic grade, histologic type).

Materials and Methods

The study was carried out on archival tissue samples collected from the Department of Pathomorphology (Jagiellonian University Medical College, Cracow). In the first two articles material consisted of 108 excisions diagnosed as primary invasive breast cancer. In the third paper 106 tissue samples were investigated. The implementation of pre-surgical systemic therapy was regarded as an exclusion factor in the study. The molecular subtype of breast cancer was determined in accordance with criteria of St Gallen classification (2013 and 2015 classification systems were applied in the first and the two latter articles, respectively). After standard fixation and embedding of tissue samples, formalin-fixed paraffin-embedded blocks were cut into 4 μm thick sections and subsequently immunohistochemistry for chymase, tryptase, CD45RO, CD20, CD56, CD8, FOXP3 and GATA3 was performed to identify investigated populations of immune cell infiltrate. Immunohistochemical staining was performed by routine manual method used in the Department of Pathomorphology. The densities of positively-stained cells were evaluated in light microscope. The numbers of mast cells, NK cells, cytotoxic and regulatory cells were expressed as a sum of positively-stained cells obtained from 5 power fields at magnification of 400x (which represented area of 1 mm^2 of tissue), either within islets of neoplastic cells or in adjacent tissue. At the same locations the percentages of tissue area occupied by T and B cells were assessed and expressed as an averaged value obtained from 5 power fields at magnification of 100x. In addition, for cytotoxic, regulatory and Th2 cells we assessed their percentages in mononuclear cell infiltrate, located in immediate surrounding of tumor, and averaged their values from 5 power fields at

magnification 400x. The obtained results were referred to clinicopathological data and subjected to statistical analysis with the use of Statistica software.

Results and conclusions

We observed the differences in densities of the investigated cells with regard to the molecular subtype of breast cancer. For tryptase- and chymase-positive mast cells, significantly higher infiltrate of these cells were noted in post-hoc test within neoplastic tissue of luminal A and B tumors, as compared to non-luminal HER2-overexpressing lesions and triple-negative breast cancers (TNBC). Inverse relationship was noted for lymphoid cells; out of all subtypes, luminal A tumors were characterized by the lowest density of T cells, both in intratumoral area (significant difference in comparison with TNBC tumors) and at tumor edge (significant differences were observed as compared to TNBC, non-luminal HER2-overexpressing and luminal B/ HER2-overexpressed cancers). Moreover, in post-hoc analysis significantly less intensive B-cell infiltrate was found at the invasion front of luminal A lesions than in TNBC and HER+ non-luminal tumors. Likewise, luminal A and B phenotypes were associated with lower density of NK cells located at invasive edge in comparison with TNBC tumors. Cytotoxic and regulatory cells observed at tumor edge were significantly more numerous in non-luminal HER2-positive and TNBC than in luminal A cancers; for regulatory cells, the latter relationship was noted within islets of cancer cells either. Additionally, density of regulatory cells located at the tumor edge differed between luminal A and B subtypes, with their higher numbers in the latter one. Higher regulatory/ Th2 cell percentages ratio was observed in infiltrates of non-luminal HER2-overexpressing tumors as compared to luminal A cancers.

Relationship between increased densities of immune cells and HER2-overexpression was found for T cells, B cells, regulatory and cytotoxic cells, exclusively at tumor edge. An inverse relationship was shown for chymase- and tryptase-positive mast cells of invasion front.

Positive correlations between ER and PR expressions were obtained for chymase- and tryptase-positive mast cells as well as for the percentage of Th2 cells in immune infiltrate and cytotoxic/ Th2 cell percentage ratio; negative correlations were observed for T cell, B cell, NK cell, cytotoxic cell and regulatory cell (both regarding their numbers and percentages in immune infiltrate) densities, as well as for regulatory/ Th2 cell percentage ratio. Concerning tumor proliferation index, we observed an inverse relationship between Ki67 protein expression in tumor cells and numbers of chymase- and tryptase-positive mast cells; positive correlation was found for T cell, B cell, NK cell and cytotoxic cell densities, as well as for regulatory cell level

(both in their number and percentage in immune infiltrate) and regulatory/ Th2 cell percentage ratio.

Higher histologic grade of breast cancer was associated with lower numbers of tryptase-positive mast cells and intratumoral chymase-positive mast cells, as well as with more dense T and B cell infiltrate, more numerous NK cells at invasion front of the tumor, higher quantities of cytotoxic and regulatory cells, increased percentage of regulatory cells in tumor-surrounding infiltrate and higher regulatory/ Th2 percentage ratio.

Invasive breast tumors of diameter larger than 2 cm (pT>1) were characterized by less numerous tryptase-positive mast cells, as well as higher T cell density, more cytotoxic cells located intratumorally and higher regulatory/ Th2 cell percentage ratio in tumor-surrounding immune infiltrate. In addition, for tumors of luminal phenotype we observed relationship between greater tumor size and more dense intratumoral cytotoxic and regulatory cells, increased regulatory cell percentage in tumor-surrounding infiltrate as well as with higher regulatory/ Th2 percentage ratio.

We observed that the incidence of breast cancer metastases in lymph nodes was related to higher percentage of regulatory cells and regulatory/ Th2 cell percentage ratio in tumor surrounding stroma. For non-luminal tumors, higher cytotoxic/ regulatory cell number ratio was noted in intratumoral area of metastatic tumors.

With regard to the histologic type of breast cancer, tumor classified as no-otherwise specified (NOS) tumors were characterized by less numerous tryptase-positive mast cells within cancer cell islets, more intensive T cell, B cell and cytotoxic cell infiltrates, more dense regulatory cells at invasive edge (both regarding cell number and their percentage in tumor-associated immune infiltrate) as well as decreased intratumoral cytotoxic/ regulatory cell number ratio in comparison with cancers of lobular morphology.

The results indicate differences in composition of tumor microenvironment in various molecular subtypes of invasive breast cancers as well as with regard to the incidence of other prognostic and predictive factors in this malignancy.

- Differences in densities of selected immune cells and their correlations with ER, PR and Ki67 expressions point to relationship between mast cell infiltrate and the incidence of clinically less aggressive breast cancer subtypes, while more abundant lymphoid cell infiltrate and increased regulatory/ Th2 cell percentage ratio were related to subtypes of more adverse clinical course;

- Differences in densities of analyzed cells indicate mast cell association with more favorable prognostic factors, such as smaller tumor size, lower histologic grade; the opposite was observed for lymphoid, cytotoxic and regulatory cells;
- Distinct relationships observed for immune cell populations located either intratumorally or at invasion front suggest distinct role of this cells in pathogenesis of breast cancer with reference to location in tumor tissue;
- Differences in densities of respective T cell subpopulations observed in luminal and non-luminal invasive breast tumors of various stage (i.e. tumor size, nodal involvement) indicate distinct role of immune cells in tumor progression depending on breast cancer molecular subtype.

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ORIGINAL ARTICLE

The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment

Anna Glajcar[1] · Joanna Szpor[1] · Agnieszka Pacek[1] · Katarzyna Ewa Tyrak[2] · Florence Chan[1] · Joanna Streb[3] · Diana Hodorowicz-Zaniewska[4] · Krzysztof Okoń[1]

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Abstract Mast cells (MCs) are a part of the innate immune system. The MC functions toward cancer are partially based on the release of chymase and tryptase. However, the MC effect on breast cancer is controversial. The aim of our study was to investigate the presence of MCs in breast cancer tumors of different molecular subtypes and their relationships with other pathological prognostic factors. Tryptase- and chymase-positive mast cell densities were evaluated by immunohistochemistry in 108 primary invasive breast cancer tissue samples. Positive cells were counted within the tumor bed and at the invasive margin. For all analyzed MC subpopulations, we observed statistically significant differences between individual molecular subtypes of breast cancer. The significantly higher numbers of intratumoral chymase- and tryptase-positive mast cells were observed in luminal A and luminal B tumors compared to triple-negative and HER2+ non-luminal lesions. A denser MC infiltration was associated with lower tumor grade, higher ER and PR expression, lower proliferation rate as well as the lack of HER2 overexpression. The results obtained in our study indicate a possible association of chymase- and tryptase-positive MCs with more favorable cancer immunophenotype and with beneficial prognostic indicators in breast cancer.

✉ Krzysztof Okoń
k.okon@uj.edu.pl

[1] Department of Pathomorphology, Jagiellonian University Medical College, ul. Grzegórzecka 16, 31-531 Kraków, Poland

[2] 2nd Department of Internal Medicine, Jagiellonian University Medical College, Kraków, Poland

[3] Department of Oncology, Jagiellonian University Medical College, Kraków, Poland

[4] Department of General, Oncological, and Gastrointestinal Surgery, Jagiellonian University Medical College, Kraków, Poland

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Introduction

Breast cancer is the most common cancer in females in the developed world. It is heterogeneous in terms of prognosis, morphology, and molecular biology; on the basis of its gene expression pattern, four main molecular subtypes were distinguished: luminal A, luminal B, HER2 non-luminal, and basal-like. This classification may be emulated by an immunohistochemical panel, which became a standard in routine pathology [1, 2].

Tumor microenvironment consists of fibroblast, endothelial, and immune cells as well as extracellular matrix (ECM) in the immediate surroundings of cancer. It influences anti-tumor host defense, tumor development, neoangiogenesis, and metastatic propensity, and may affect patient's outcome [1–3].

Mast cells (MCs) are bone marrow-derived cells commonly associated with allergic reactions and responses to parasitic infestations. MC granules store numerous mediators, including heparin, histamine, proteases, chemokines, and growth factors, which are released upon MC activation and contribute to tissue repair, wound healing, and angiogenesis. They modulate functions of other immune cells by either enhancing immunologic response or inducing immune tolerance. MCs are also one of the first cells to infiltrate cancer and can either promote or suppress tumor growth [4–8].

Proteases constitute approximately one fourth of MCs protein content. Based on the expression of chymase and tryptase, the mast-cell-specific serine proteases, human MCs are divided into MC_T, which expresses only tryptase and MC_{TC}, which expresses both tryptase and chymase. These populations predominate in different

anatomical locations and vary according to their functions [4, 6, 9]. Tryptase participates in ECM remodeling and is a potent proangiogenic factor, in part by protease-activated-receptor 2 (PAR-2) activation [9–11]. MC tryptase was also reported to activate tumor-associated fibroblasts [12]. Chymase is thought to be important mainly for ECM remodeling; however, it may also induce angiogenesis by activating metalloproteinases (MMPs), such as MMP-9, which releases proangiogenic mediators from stroma [6, 12, 13].

Some MC proteases are stored in complexes with heparin [14]. Heparin suppresses proliferation and reduces the number of breast cancer cell colonies. It was hypothesized that heparin might interrupt interactions between tumor-associated fibroblasts and cancer cells, thus impairing tumor development [15].

The aim of the study was to investigate the density of MCs expressing tryptase and chymase in breast cancers of different molecular subtypes and to examine their relationships with more standard prognostic factors.

Preliminary results from this study were presented at the 6th Jagiellonian University Medical College Doctoral Students' Conference.

Materials and methods

Materials

The material consisted of routinely processed, formalin-fixed paraffin-embedded primary invasive breast carcinomas diagnosed between 2002 and 2014. The archival hematoxylin–eosin-stained slides were re-evaluated and representative, well-preserved specimens were chosen for immunohistochemistry. For nuclear grading, Nottingham Histologic Grade system was used, while staging was performed according to 2010 AJCC system [16].

Immunohistochemistry

Immunohistochemistry for tryptase, chymase, estrogen receptor (ER), progesterone receptor (PR), and Ki67 protein was performed according to the protocol routinely used in our laboratory. The selected blocks were cut into 4-μm-thick sections. Antigen retrieval was performed by incubating the slides in citrate buffer (pH 6.0; 0.01 M) or EDTA (pH 8.0; 0.01 M) at 97 °C in a water bath for 40 and 30 min, respectively, or by enzymatic digestion with proteinase (21 °C, 7 min). Primary antibodies used in the study are listed in Table 1.

UltraVision Quanto detection system (LabVision; ThermoScientific, USA) and 3,3′-diaminobenzidine as chromogen were used, and the slides were counterstained with Mayer hematoxylin (Thermo Fisher Scientific, Waltham, USA) and coverslipped.

Immunohistochemistry for HER2 was performed on BenchMark BMK Classic autostainer (Ventana, USA) using UltraVIEW DAB Detection Kit (Ventana Medical Systems Inc., USA).

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

Table 1 Antibodies used in the study

	Clone	Dilution	Antigen retrieval	Incubation time (min)	Manufacturer
Tryptase	AA1	1:100	Proteinase	60	Novocastra (Leica Biosystems, Germany)
Chymase	CC1	1:100	Citrate	30	LabVision (ThermoScientific, USA)
Estrogen receptor	6F11	1:25	Citrate	60	Novocastra (Leica Biosystems, Germany)
Progesterone receptor	PgR636	1:50	Citrate	60	Dako, USA
Ki67	MIB-1	1:100	EDTA	60	Dako, USA
HER2/neu	PATHWAY4B5				Ventana Medical System Inc., USA

Evaluation of immunostaining

The slides stained for tryptase and chymase were scanned on Nikon Labophot-2 optical microscope (Tokyo, Japan) at low magnification (×100), and the areas with the highest number of positive cells were chosen. Then, positively stained cells were counted in five high-power fields (HPF) (400 × 0.2 mm^2 field area), which represented 1 mm^2 of the examined tissue. The positive cells located no further than 1 HPF from the tumor edge were regarded as invasive margin, while positive cells located within neoplastic tissue further than 1 HPF from the tumor edge inwards were considered as intratumoral population.

Positive ER and PR expression were set when ≥1% of neoplastic cells showed positive immunostaining. The threshold for discriminating between low and high Ki67 expression was set at ≥14% of positive cells. Scoring of the HER2 stain was performed by standard method [17].

Definition of breast cancer molecular subtypes

The cases were classified into molecular subtypes according to St Gallen 2013 International Expert Consensus: luminal A (ER+ and PR ≥20%, Ki67 < 14%, HER2−), luminal B/ HER2 − (ER+, HER2− with PR <20% and/or Ki67 ≥ 14%), luminal B/HER2+ (ER+ or PR+, HER2+), HER2+ non-luminal (ER −/PR−/HER2+), and triple-negative breast cancer (ER−/PR −/HER2−) [18].

Statistical analysis

To assess the differences in positive cells' infiltrate between groups, ANOVA Kruskal–Wallis and Mann–Whitney *U* tests were performed. The correlations between groups were evaluated by using Spearman rank test. All analyses were performed using Statistica 10 (StatSoft Inc., USA). p values <0.05 were considered statistically significant.

Results

Study group

The study group consisted of 108 cases. The mean age of patients at the time of diagnosis was 55.3 years, ranging from 29 to 87 years. Sixty cases (55.5%) were stage pT1, 45 cases (41.7%) pT2, and 3 cases (2.8%) pT3. Lymph node status was pN0 in 54 cases (50.0%), pN1 in 31 cases (28.7%), pN2 in 9 cases (8.3%), and pN3 in 13 cases (12.0%).

Distribution of molecular subtypes was as follows: luminal A in 30 cases (27.8%), luminal B/HER2− in 19 cases (17.6%), luminal B/HER2+ in 10 cases (9.3%), HER2+ non-luminal (HER2+) in 20 cases (18.5%), and triple-negative breast cancer (TNBC) in 29 cases (26.8%). On the basis of the histologic type, 91 cases (84.3%) were classified as "not otherwise specified" (NOS), 15 cases (13.9%) as lobular, and 2 cases (1.8%) as "other." Nottingham Histologic Grade was G1 in 17 cases (15.7%), G2 in 37 cases (34.3%), and G3 in 54 cases (50%). The patients and tumor characteristics are shown in Table 2.

MC subpopulations in different breast cancer subtypes

First, we investigated whether the mast cell counts differed between cancers of luminal (ER+ or PR+) and non-luminal (ER− and PR−) immunophenotype. A statistically significant difference was observed for both chymase- and tryptase-positive MCs in either intratumoral location or at the invasive margin (Fig. 1). In all cases, the luminal subtype of tumors was associated with relatively higher MC count (Table 3).

Thorough analysis of each of the investigated MC populations showed significant differences in the density of infiltration between molecular subtypes of cancer; this was most evident for intratumoral cells. The number of intratumoral

Table 2 Clinicopathologic features of the study group

Characteristic	Number of cases	Percent
Age		
Range: 29–87		
Mean: 55.3		
Tumor size		
pT1	60	55.5
pT2	45	41.7
pT3	3	2.8
Lymph node status		
pN0	54	50.0
pN1	31	28.7
pN2	9	8.3
pN3	13	12.0
Nottingham Histologic Grade		
G1	17	15.7
G2	37	34.3
G3	54	50.0
Histological type		
Ductal	91	84.3
Lobular	15	13.9
Other	2	1.8
Molecular subtype		
Luminal A	30	27.8
Luminal B	19	17.6
Luminal B/ HER2+	10	9.3
HER2+ non-luminal	20	18.5
Triple negative	29	26.8

Table 3 MC densities in breast cancers of different molecular subtype, immunophenotype, Ki67, and HER2 expression

	Chymase				Tryptase			
	Intratumoral		Invasive margin		Intratumoral		Invasive margin	
	Mean (SD)	*p*	Mean (SD)	*p*	Mean (SD)	*p*	Mean (SD)	*p*
Molecular subtype								
Luminal A	22.59 (10.38)	<0.001	23.21 (7.87)	<0.025	40.40 (16.97)	<0.001	36.27 (20.62)	<0.015
Luminal B	27.72 (12.76)		25.68 (11.95)		36.94 (17.56)		38.74 (19.97)	
Luminal B/HER2+	20.80 (12.45)		19.50 (7.55)		31.10 (20.89)		26.70 (11.49)	
HER2+ non-luminal	15.30 (7.89)		18.70 (9.76)		25.90 (11.43)		24.65 (9.24)	
Triple negative	14.56 (9.82)		19.03 (7.35)		22.50 (13.42)		27.17 (11.28)	
Immunophenotype								
Luminal	23.89 (11.64)	<0.001	23.38 (9.43)	<0.005	37.72 (17.86)	<0.001	35.44 (19.34)	<0.004
Non-luminal	14.87 (8.97)		18.90 (8.32)		23.92 (12.61)		26.14 (10.46)	
HER2 overexpression								
No	20.91 (11.88)	NS	22.25 (9.15)	<0.025	32.99 (17.69)	NS	33.49 (18.01)	<0.015
Yes	17.13 (9.79)		18.97 (8.96)		27.63 (15.08)		25.33 (9.89)	
Ki67 expression								
Low	22.41 (10.97)	NS	22.79 (8.27)	NS	39.17 (16.29)	<0.001	34.89 (19.61)	NS
High	18.56 (11.47)		20.43 (9.44)		27.68 (16.28)		29.22 (14.62)	

chymase-positive MCs was the highest in luminal B cancers, which differed significantly from TNBC ($p < 0.002$) and HER2+ non-luminal ($p < 0.025$) tumors. Luminal A cancers contained significantly more chymase-positive MCs than TNBC cancers ($p < 0.04$). The intratumoral tryptase-positive MC density was the highest in luminal A tumors and was significantly higher than that in TNBC ($p < 0.001$) and HER2+ non-luminal ($p < 0.04$) cases. The abundance of these cells was also significantly higher in luminal B as compared to TNBC tumors ($p < 0.015$). There was a significant difference in MC density at the invasion front between all the molecular breast cancer subtypes, but no significant difference in post hoc analysis was observed (Fig. 2, Table 3).

The number of MCs at the invasive margin, either chymase- or tryptase-positive, was significantly increased in tumors without HER2 overexpression ($p < 0.025$ and $p < 0.015$, respectively) compared to that in HER2 overexpressed tissues (Table 3).

We also observed also that intratumoral tryptase-positive MCs were strongly associated with tumors of low Ki67 expression ($p < 0.001$) (Table 3).

The numbers of all investigated MC populations showed significant positive correlations with ER and PR expression, as well as a negative correlation with mitotic index. For investigated subpopulations, either in intratumoral area or at the invasion edge, tryptase-positive MCs correlated negatively with Ki67 expression. However, for chymase-positive MCs, such correlation was observed only within the tumor bed.

MC subpopulations and other pathological prognostic factors

Investigated tumors were stratified according to their size into tumors of diameter ≤2 cm (pT1) and >2 cm (pT > 1). We observed statistically significant differences in tryptase-positive cell densities in both intratumoral compartment

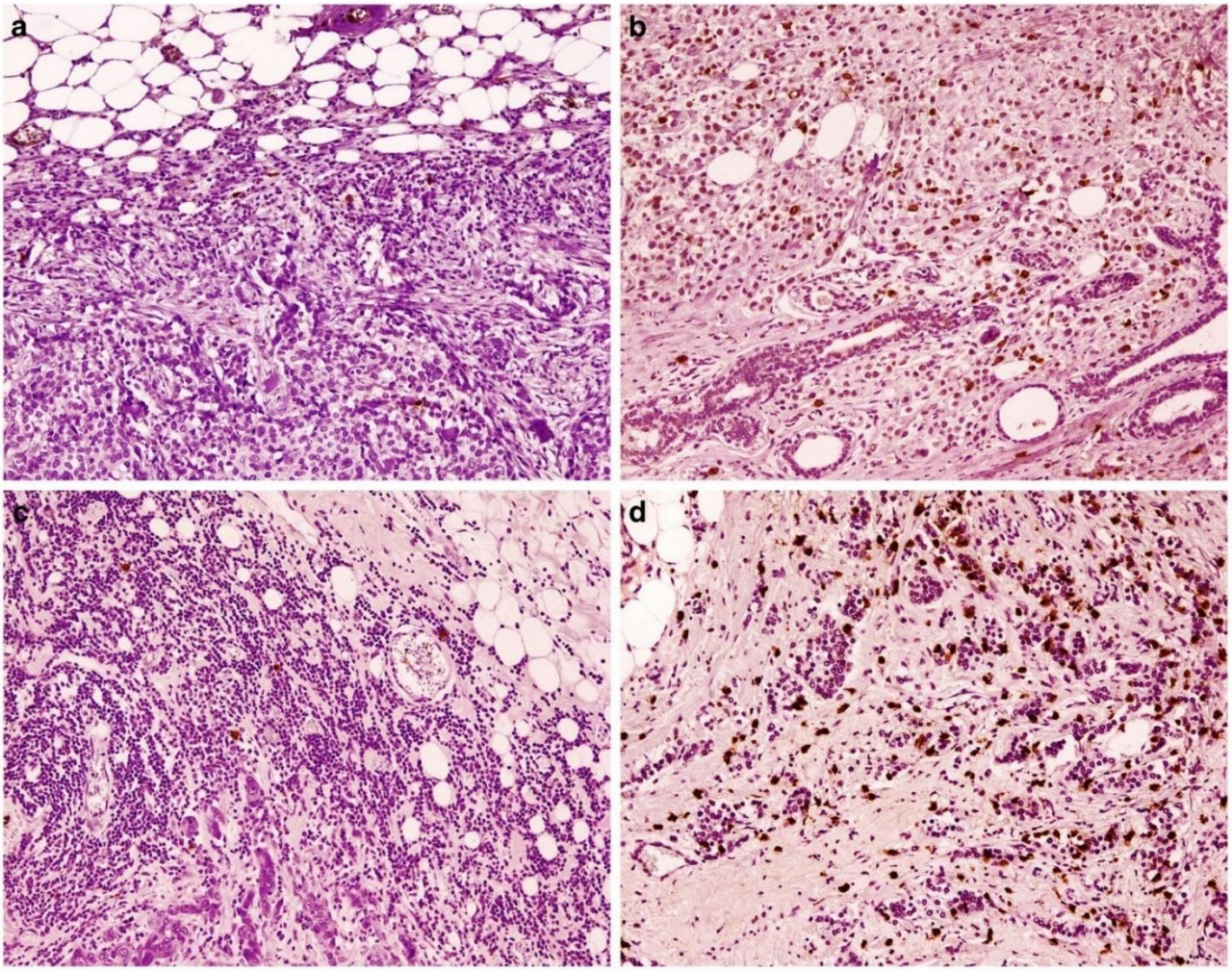

Fig. 1 Mast cells in invasive breast cancer. Low (**a**) and high (**b**) chymase-positive mast cells infiltration, low (**c**) and high (**d**) tryptase-positive mast cells infiltration. Immunohistochemistry for tryptase and chymase, magnification ×100

($p < 0.008$) and at the invasion front ($p < 0.02$) between the two groups. The intratumoral tryptase-positive MC density was higher in pT1 tumors (mean 35.5, SD 18.2) as compared to pT > 1 lesions (mean 26.6, SD 14.3). Similarly, for tryptase-positive MCs at the tumor margin, higher density was observed in smaller-sized cancers (pT1—mean 33.7, SD 17.2; pT > 1—mean 28.1, SD 15.2). There were no statistically significant differences in MC densities between cases with and without nodal involvement.

There were significant differences in the densities of tryptase-positive cells, both in intratumoral compartment and at the invasive margin, as well as intratumoral chymase-positive cell count between tumors of different Nottingham Histologic Grades. The number of intratumoral chymase-positive cells was significantly higher in G1 ($p < 0.015$) and G2 ($p < 0.008$) tumors as compared to G3 lesions. Tryptase-positive MC densities for both intratumoral compartment and invasion front were significantly higher in G1 than in G3 cancers ($p < 0.015$ and $p < 0.05$, respectively) (Fig. 3, Table 4).

In respect of tumor histological type, intratumoral tryptase-positive cells were significantly associated with lobular phenotype (Table 4).

Discussion

The studies concerning MC infiltration in various breast cancer molecular subtypes were scarce and the results were encumbered by varied subtype classifications. In our study, we noted that chymase- and tryptase-positive MC infiltration differed between breast cancers of respective molecular subtypes in both intratumoral area as well as at the invasive margin, and that higher MC numbers were associated with less aggressive cancer types. Similar to our results, della Rovere et al. observed high MC density in breast cancer expressing high levels of hormone receptors. As a result, the authors considered MC infiltration in this neoplasm as a protective factor against tumor progression, potentially due to MC cytolytic activity against malignant cells

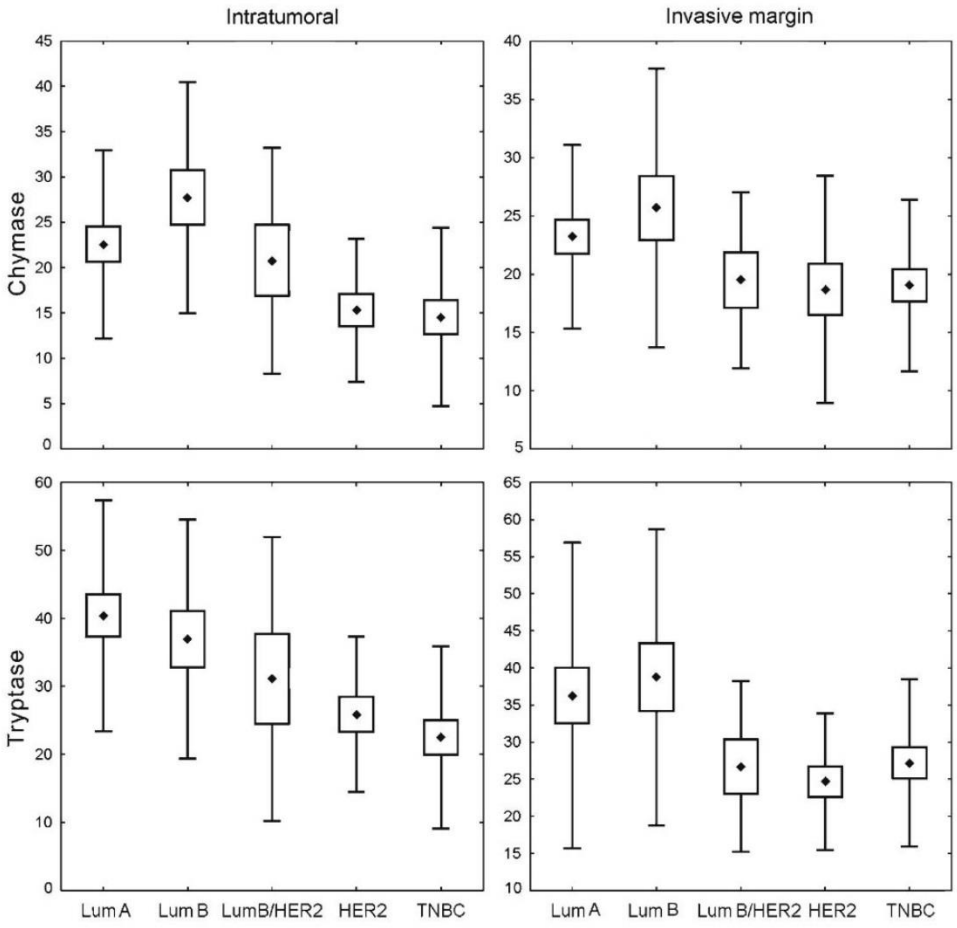

Fig. 2 Density of investigated MC subpopulations in breast cancer specimens representing different molecular subtypes: *Lum A* luminal A, *Lum B* luminal B/HER2−, *Lum B/HER2* luminal B/HER2+, *HER2* HER2+ non-luminal, *TNBC* triple-negative subtype. *Central point* is the arithmetic mean, *box* is the arithmetic mean ± standard error, and *whisker* is the arithmetic mean ± standard deviation. ANOVA Kruskal–Wallis test, *p*-values are shown in Table 3

[19, 20]. Raica et al. noted that density of intratumoral, but not peritumoral, tryptase-positive MCs was higher in luminal A, luminal B, and HER2-positive breast cancers compared to basal-like breast cancers [21]. This was partially analogous to our results, which suggested that non-luminal HER2-positive subtype was associated with low tryptase-positive MC content. In

Table 4 MC density according to tumor grade and breast cancer histological type

	Chymase				Tryptase			
	Intratumoral		Invasive margin		Intratumoral		Invasive margin	
	Mean (SD)	*p*	Mean (SD)	*p*	Mean (SD)	*p*	Mean (SD)	*p*
Nottingham Histologic Grade								
1	24.41 (10.24)	<0.002	23.06 (8.67)	NS	39.65 (17.93)	<0.007	34.53 (11.46)	<0.02
2	23.46 (11.81)		22.36 (9.68)		34.44 (17.59)		36.19 (22.92)	
3	15.87 (10.22)		20.09 (8.98)		26.83 (15.26)		26.78 (10.72)	
Histological type								
NOS	19.07 (11.12)	NS	20.69 (8.57)	NS	30.56 (17.14)	<0.05	29.48 (11.97)	NS
Lobular	24.79 (11.65)		25.71 (12.28)		38.73 (15.93)		42.93 (31.32)	

the quoted study, significant correlations between peritumoral tryptase-positive MCs and lymphatic microvessel densities were found in luminal A and basal-like cancers. Such observation might indicate MC involvement in lymphangiogenesis and lymphovascular spreading of breast cancer, particularly of luminal A type [21]. Other studies also outlined the correlation between tryptase-positive MCs and microvessel density in breast cancer [22, 23].

We observed that all analyzed populations of MCs correlated positively with ER and PR expression and negatively with mitotic index. Additionally, tryptase-positive MCs both of the intratumoral area and at the invasion front were negatively associated with tumor size, while tryptase-positive as well as intratumoral chymase-positive MCs showed an inverse correlation with Ki67 expression. These findings supported the aforementioned hypothesis of the protective role assumed by MCs against cancer progression. Similarly, other studies also suggested a negative correlation of tryptase-positive MCs with tumor size [24], along with a positive correlation with PR [25] and ER expression [24]. Although several studies failed to show independent prognostic significance of MCs in breast cancer [24–26], and few works have even shown that peritumoral MC infiltration was associated with poor short-term survival [27], MCs were still proposed by some authors as an additive favorable prognostic factor [19, 25]. It was further postulated that even a single MC in tumor surrounding might have a beneficial impact on the prognosis [28]. Rajput et al. observed a positive but not significant correlation between MCs and HER2 expression [28]. However, our study suggested that chymase- and tryptase-positive MC densities at the tumor front were associated with tumors that did not indicate HER2 overexpression. Some other studies observed an inverse correlation between tryptase-positive MCs and Ki67 expression [24], while others did not [19]. Contrary to our results and the aforementioned literature, Ranieri et al. did not find any associations between MC number and tumor size, histological grade, ER/PR status, or HER2 overexpression in early breast cancer [23].

Our study indicated that low- and intermediate-grade breast cancers contained high numbers of MCs in both intratumoral location and at the invasive margin. In consistence with our results, some authors reported that tryptase-positive MCs correlated negatively with tumor histological Elston grade [24, 25]. A plausible explanation could be that low-grade breast cancer elicited more effective innate immune response, or that high-grade cancer suppressed such response. Strikingly, Xiang et al. observed more numerous peritumoral MCs in G3 breast cancers than lower grades, and reported more intensive tryptase immunostaining in the surrounding of node-positive tumors as compared to node-negative ones. In this experimental study, tryptase itself did not increase proliferative activity of breast cancer cell lines. However, in the presence of heparin, tryptase increased cancer cell migration and expression of activated MMP-1. As tryptase was activated by

Table 5 Immunohistochemical studies, which evaluated mast cells in breast cancer

Authors	Material	Mast cells' marker	Conclusions	Reference
Bowers H. et al., 1979	Axillary lymph nodes of 43 breast cancer patients	Toluidine blue	Higher MC number is associated with better patients' survival	[30]
Samoszuk M., Corwin M., 2003	35 breast cancer tissue sections of varying stages	Tryptase	A tendency toward peritumoral accumulation of MCs in preinvasive and intratumoral accumulation in invasive tumors	[31]
Amini RM. et al., 2007	234 invasive breast cancer tissues	Tryptase	MCs are associated with estrogen receptor positivity and low tumor grade	[25]
della Rovere F. et al., 2007	50 cases of invasive ductal breast cancer	Alcian blue	Higher MC content is associated with high hormone-receptive cancers	[19]
Ribatti D. et al., 2007	80 sentinel lymph nodes of breast cancer patients	Tryptase	Higher MC number in micrometastatic lymph nodes; MC quantity increases with angiogenesis	[32]
Rajput A. et al., 2008	4444 invasive breast cancer tissues	CD117	Presence of MCs in tumor stroma associated with better patients' survival	[28]
Ranieri G. et al., 2009	88 breast cancer patients' biopsy specimens	Tryptase	MCs are associated with angiogenesis	[23]
Xiang M. et al., 2010	80 breast cancer tissues	Tryptase	MC number positively correlated with tumor grade and was associated with nodal involvement	[29]
Löfdahl B. et al., 2012	190 lymph-node-negative breast cancer tissue samples	Tryptase	Negative associations between MC number and adverse prognostic factors	[24]
Raica M. et al., 2013	55 ductal invasive breast cancer tissues	Tryptase	Interplay between MCs and lymph vessels is specific for each molecular subtype of breast cancer	[21]
Marech I. et al., 2014	105 cases of breast cancer	Tryptase	Mast cell tryptase is involved in angiogenesis	[22]

low pH and heparin, the authors concluded that tryptase promoted metastatic spread after microcirculation failed to remove acidic substances. This could potentially explain the higher MC count in more aggressive, more rapidly growing, grade 3 carcinomas observed in the study [29]. The findings from other immunohistochemical studies in breast cancer are summarized in Table 5.

Although the role of MCs in breast cancers has been investigated by several authors, the obtained results appeared to be ambiguous. Roy et al. [33] used an experimental model of arthritic mice for their study, which showed an elevated number of MCs within primary mammary tumors and at the sites of metastasis in comparison with the control group. This may be explained by the increased MC migration toward tumor and their activation within malignant lesion. MCs were suggested to attract stem cell factor (SCF) expressing breast cancer cells, thus facilitating the spread of the tumor. As SCF/c-kit signaling is considered to be one of the most potent chemoattractants and activators of MCs, SCF-positive neoplastic cells contributed, in turn, to subsequent infiltration, differentiation, and survival of MCs, which would eventually enhance metastatic potential of breast cancer [33]. Samoszuk et al. reported that MCs could counteract tumor hypoxia by releasing anticoagulants, which improved the blood flow. The authors also noted that tryptase-positive MCs in early breast cancer were more abundant in peritumoral stroma, while in invasive tumors, MCs were more extensively located within tumor tissue [31]. In the skin of breast cancer patients, chymase- and tryptase-positive MCs increased collagen production by interacting with dermal fibroblasts [34, 35]. It was also shown that MC tryptase has the capability to modify breast cancer microenvironment by converting fibroblasts into activated myofibroblasts, which, in turn, may promote tumor development. However, the accumulation of degranulated MC_T at the invasion margin was interpreted as an evidence for protective role against cancer growth [36]. Bowers et al. observed significantly higher MC number in axillary lymph nodes of breast cancer patients who survived for longer than 60 months post-mastectomy, in comparison with patients with a shorter survival time span. As a result, the authors postulated that MCs might be involved in host tumor resistance [30]. In contrast, higher MC and microvessel counts in sentinel lymph nodes with micrometastases as compared to non-metastatic sentinel lymph nodes could suggest the participation of MCs in metastasis formation [32].

Mast cells were also investigated in other types of cancer. The MC count in squamous cell carcinoma of the lip was found to be higher compared to that in normal tissue. The distribution of MCs in this neoplasm differed with reference to location: within the tumor nest, MC_T prevailed over MC_{TC} cells, while MC_{TC} predominated at the tumor front. It was postulated that the latter might influence cancer invasion

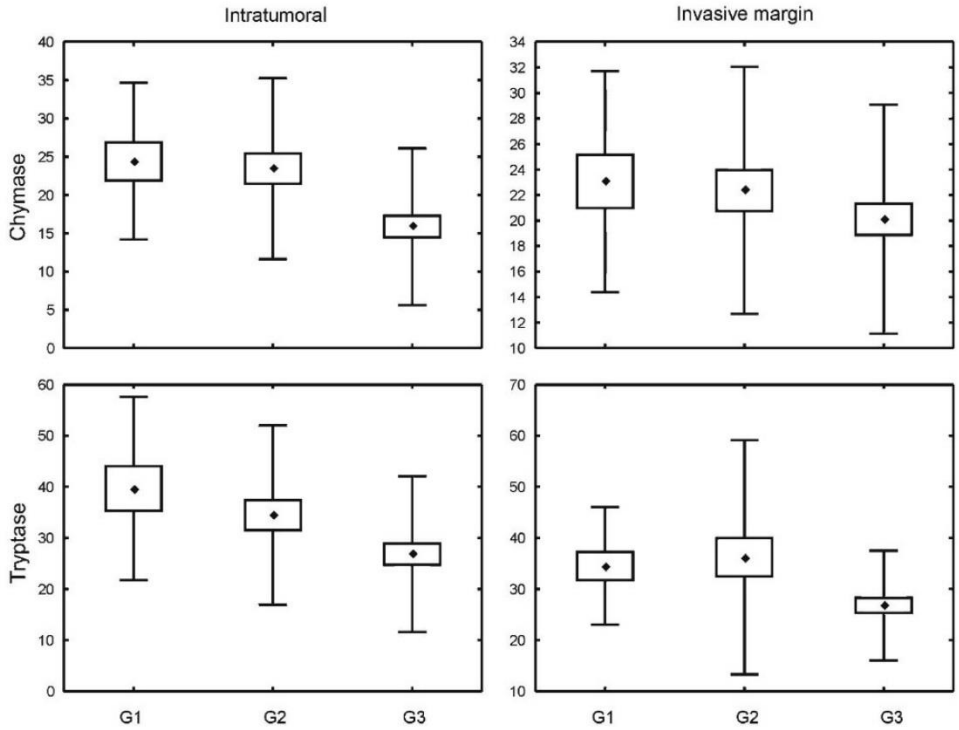

Fig. 3 Density of investigated MC subpopulations in breast cancer specimens representing different Nottingham Histologic Grade. *Central point* is the arithmetic mean, *box* is the arithmetic mean ± standard error, and *whisker* is the arithmetic mean ± standard deviation. ANOVA Kruskal–Wallis test, *p* values are shown in Table 4

[37]. Mast cells displayed different phenotypes in normal, hyperplastic, and malignant prostate tissues, thus suggesting alteration in MC phenotypes and their involvement in pathogenesis of prostate cancer. Moreover, peritumoral tryptase- and chymase-positive MCs correlated with increasing Gleason score [38]. In the cervix, the overall MC level was stable in pre-cancer, but increased significantly in invasive cancer. The prevailing phenotype of mast cells was MC_T, and the authors hypothesized that this population may stimulate neovascularization and promote tumor progression and metastasis [39]. In addition, in patients on hemodialysis with renal cell carcinoma, MC_T were also reported to predominate; an elevated SCF expression in specimens from hemodialyzed patients could potentially account for this MC_T increase. MC_T density correlated positively with proliferative index and PAR-2 expression in tumor cells [40]. Melanomas were noted to display lower numbers of both chymase- and tryptase-positive intratumoral MCs as compared to common and dysplastic nevi. Interestingly, the number of these cells increased from common to dysplastic nevi. The authors suggested that the observed decrease of MCs in malignant melanoma might be due to the self-sufficiency of this neoplasm to induce neoangiogenesis or to break the host defense barrier [41].

Several authors focused on the associations between MCs and angiogenesis, a phenomenon linked to progression in various neoplasms. In non-small cell lung carcinoma, MC_{TC} correlated with blood vessel count both inside the tumor and at the invasive margin. In contrast, MC_T number correlated with blood vessel count only at the invasive margin, potentially due to angiogenesis being associated mainly with MC_{TC} density [42]. In an experimental mice skin cancer model, de Souza et al. observed that tumor MCs were recruited to the tumor microenvironment at their immature state, and that the number of both immature and mature MCs increased parallel to cancer progression. At early phases of tumor development, tryptase promoted neoangiogenesis, while in later stages it modulated vessel growth. Both chymase and tryptase expressions increased during tumor progression, and correlated with either MC maturation or new vessel formation, indicating the involvement of these two proteases in cancer progression [43]. Similarly in gastric carcinoma, tryptase- and chymase-positive MCs increased with stage and grade, and were associated with neoangiogenesis [44]. In colorectal adenocarcinoma, tryptase-positive MCs were found mainly in the immediate vicinity of blood vessels. However, as some of the tumor vessels lacked associated inflammatory cells, it was probable that inflammatory infiltration was not required for the induction of angiogenesis [45]. In contrast, neither tryptase- nor chymase-positive MC densities were related to microvessel counts in mesothelioma, though tryptase-positive MCs were associated with a better overall survival rate and a longer time till progression [46].

As it might be inferred from the aforementioned studies, MC contribution to tumor progression was observed in many neoplasms [12, 37, 38, 41, 43, 44]. However, in some tumors, MCs were regarded as protective factor [46], with their undefined role in breast cancer [24, 25, 29]. In various cancers MC distribution [37], as well as their prognostic significance, may vary depending on MC intratumoral [40, 41] or peritumoral [38] location, and in some cancers their increasing malignancy was reported to be associated with MC phenotype alteration [37, 38]. In breast cancer, an increase in the number of non-degranulated MCs from normal to malignant tissue was observed [36]. MC functions are strongly dependent on microenvironmental factors, and both cytokines as well as hormones may affect even mature MCs and influence their number, activation, suppression, mediators' content, and phenotype [4, 47, 48]. Thus, it is not unlikely that on the basis on their functions and phenotype, in various cancers, the existence of several subpopulations of MCs could be considered, which might be partially analogous to distinction between M1 and M2 macrophages [47, 49].

In conclusion, our study outlined the associations of MCs with positive prognostic factors in breast cancer. We also observed that the breast cancer molecular subtypes differed in their chymase- and tryptase-positive MC content. However, further investigation is required to elucidate their impact on the breast cancer prognosis.

Compliance with ethical standards The study was approved by Jagiellonian University Committee of Bioethics (consent number 122.6120.149.2016).

Conflict of interest The authors declare that they have no conflicts of interest.

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Original paper

Lymphoid environment in molecular subtypes of breast cancer

Anna Glajcar[1], Joanna Szpor[1], Katarzyna Ewa Tyrak[2], Joanna Streb[3], Diana Hodorowicz-Zaniewska[4], Krzysztof Okoń[1]

[1]Chair and Department of Pathomorphology, Collegium Medicum, Jagiellonian University, Krakow, Poland
[2]II Chair of Internal Medicine, Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland
[3]Department of Oncology, Jagiellonian University Medical College, Krakow, Poland
[4]I Chair of General, Oncological, and Gastrointestinal Surgery, Jagiellonian University Medical College, Krakow, Poland

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

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

Introduction

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

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

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

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

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

Material and methods

Materials

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

Immunohistochemistry

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

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

Table I. Antibodies used in the study

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

Evaluation of immunostaining

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

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

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

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

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

Definition of breast cancer molecular subtypes

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

Results

Description of the study group

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

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

Differences in lymphocytic infiltrate between respective breast cancer molecular subtypes

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

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

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

NS – not significant

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

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

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

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

Associations between lymphocytic infiltrate and other prognostic indicators in breast cancer

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

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

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

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

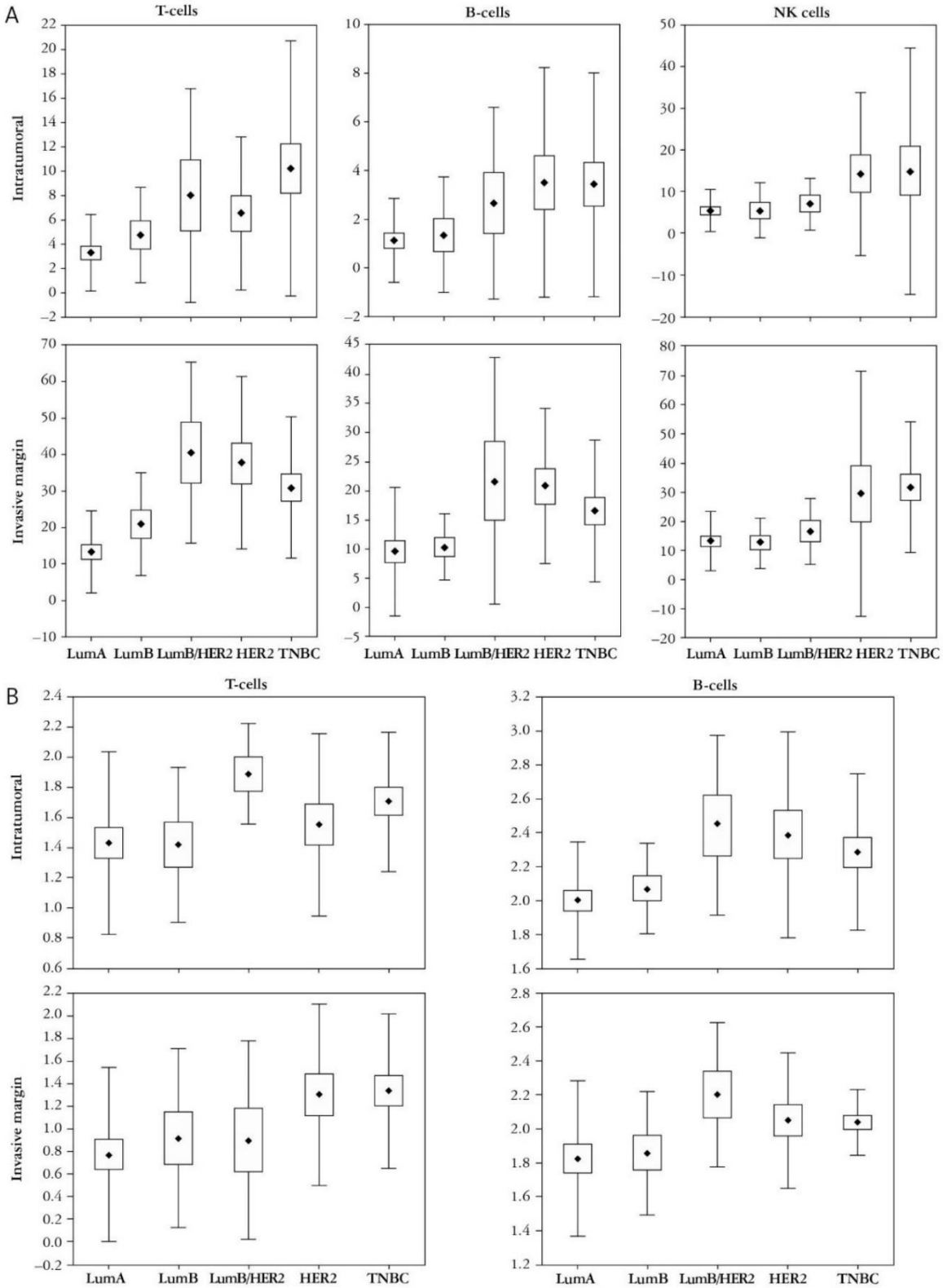

Fig. 1. The densities of T-cells, B-cells, and NK cells with reference to St Gallen 2015 molecular subtype. A) Immune cell quantities evaluated as a percentage of tumour area involved; B) Infiltration density assessed by the scoring system of Kreike *et al*. Central point is the arithmetic mean, box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

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

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

NS – not significant

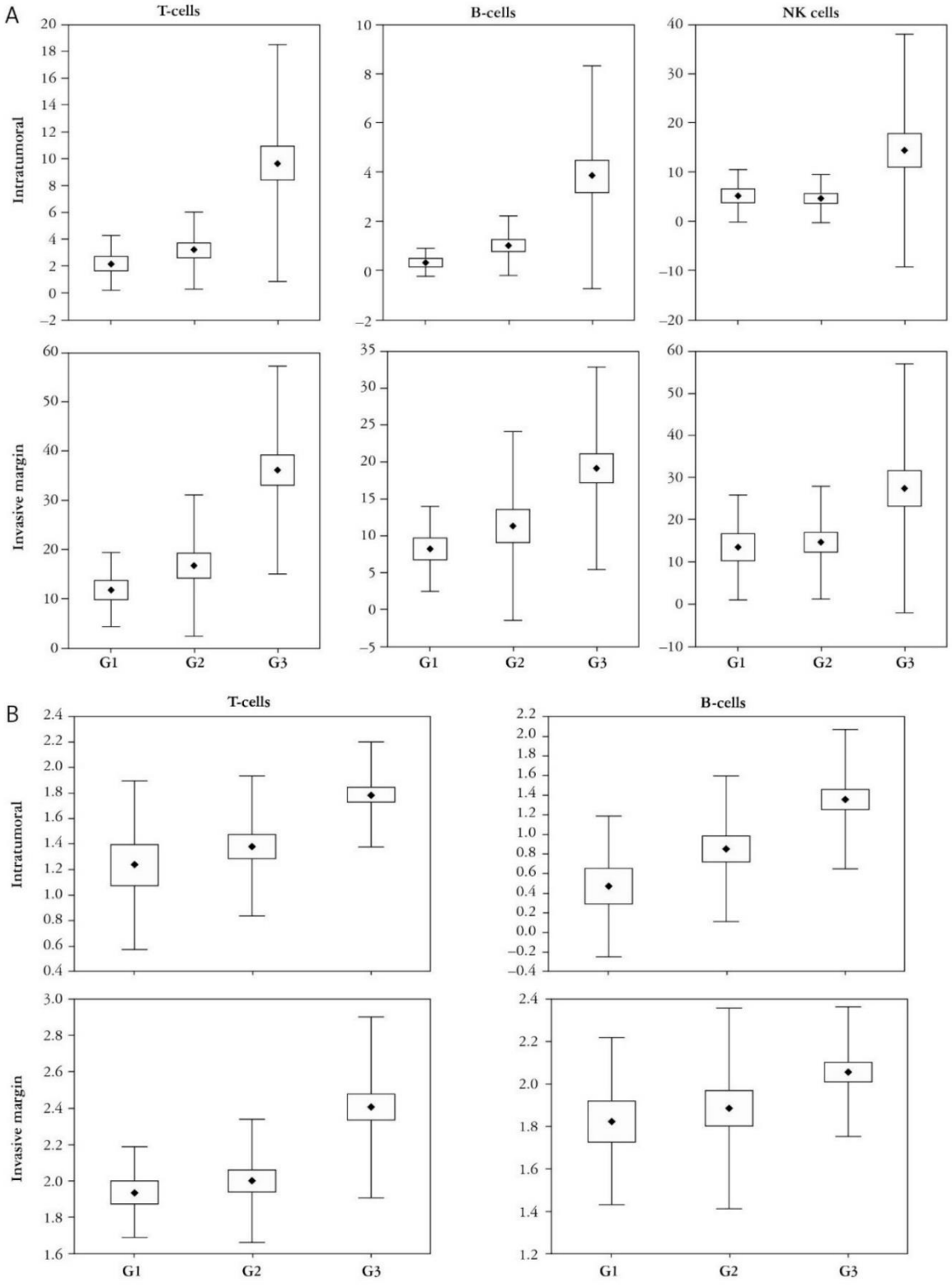

Fig. 2. The densities of T-cells, B-cells, and NK cells with reference to Nottingham Histologic Grade. A) Immune cell quantities evaluated as a percentage of tumour area involved; B) Infiltration density assessed by the scoring system of Kreike *et al*. Central point is the arithmetic mean, box is the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

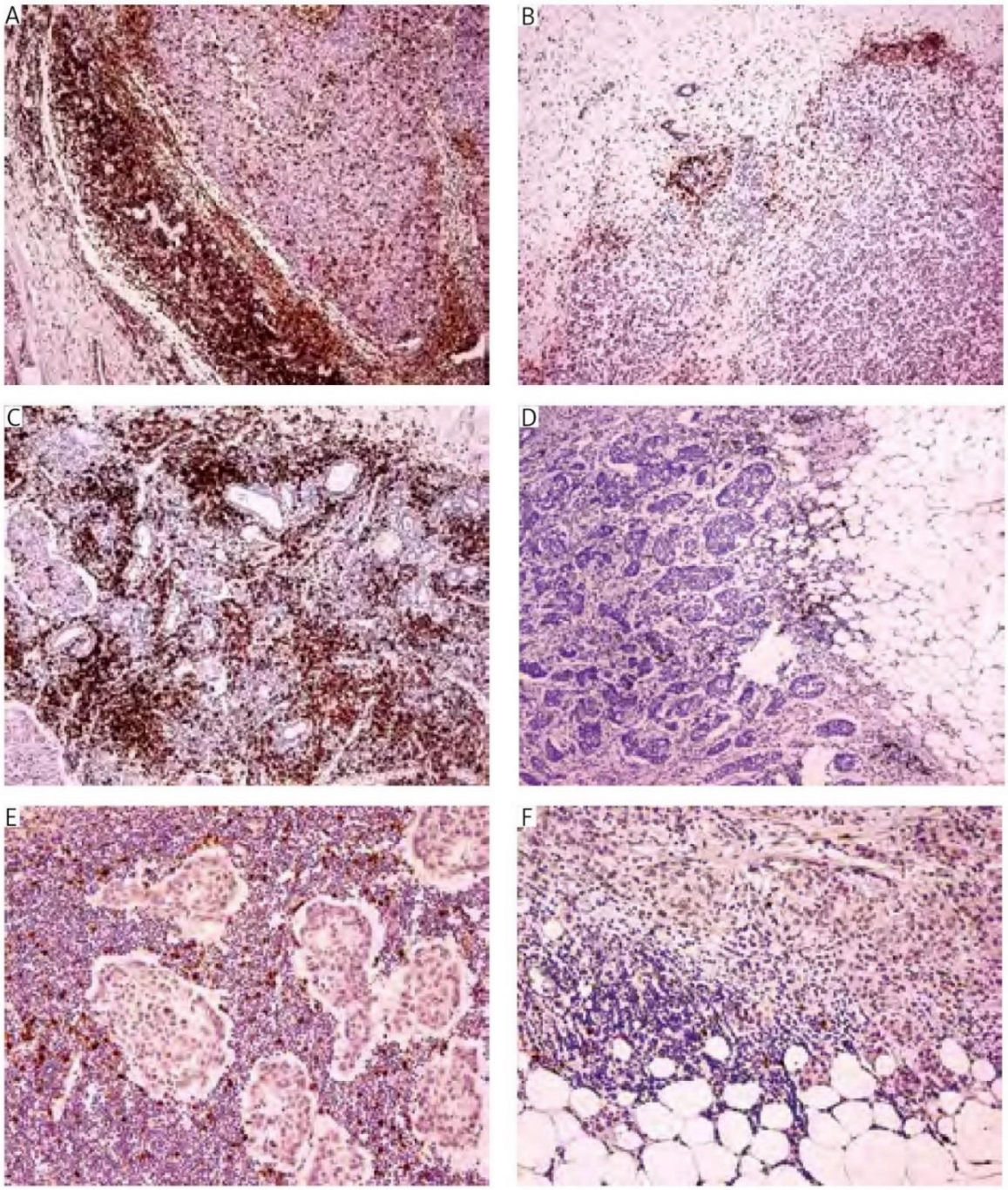

Fig. 3. Lymphoid infiltration of invasive breast cancer tissue. Abundant and low densities of T-cells (A, B), B-cells (C, D), and NK cells (E, F). Immunohistochemical staining for CD45RO, CD20, and CD56, light microscopy, magnification used: 50× (A-D) and 100× (E, F).

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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The authors declare no conflict of interest.

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Address for correspondence

Krzysztof Okoń, MD PhD
Chair and Department of Pathomorphology
Collegium Medicum
Jagiellonian University
Grzegórzecka 16
31-531 Kraków, Poland
tel. +48 12 421 15 64
fax +48 12 411 97 25
e-mail: k.okon@uj.edu.pl

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ORIGINAL ARTICLE

The composition of T cell infiltrates varies in primary invasive breast cancer of different molecular subtypes as well as according to tumor size and nodal status

Anna Glajcar[1] · Joanna Szpor[1] · Diana Hodorowicz-Zaniewska[2] · Katarzyna Ewa Tyrak[3] · Krzysztof Okoń[1]

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Abstract

T lymphocytes are the most numerous immune cells in tumor-associated infiltrates and include several subpopulations of either anticancer or pro-tumorigenic functions. However, the associations between levels of different T cell subsets and breast cancer molecular subtypes as well as other prognostic factors have not been fully established yet. We performed immunohistochemistry for CD8 (cytotoxic T cells (CTL)), FOXP3 (regulatory T cells (Tregs)), and GATA3 (Th2 cells) in 106 formalin-fixed paraffin-embedded invasive breast cancer tissue samples and analyzed both the numbers and percentages of investigated cells in tumor-associated infiltrates. We observed that triple-negative breast cancer (TNBC) and HER2+ non-luminal breast tumors were associated with more numerous CTLs and Tregs and a higher Treg/Th2 cell ratio as compared with luminal A subtype. A higher Treg percentage was related to a decreased hormone receptor expression, an increase in the Ki67 level, a greater tumor size of luminal tumors, and the presence of lymph node metastases. Moreover, differences in the composition of T cell infiltrates were associated with HER2 status and histologic grade and type, and a distinct immune pattern was observed in tumors of different phenotypes regarding pT stage and nodal status. The results of our work show the diversity of T cell infiltrates in primary invasive breast cancers of different phenotypes and suggest that progression of luminal or non-luminal tumors is related to distinct tumor-associated T cell composition.

Keywords Breast cancer · Microenvironment · Cytotoxic T cells · Regulatory T cells · Th2 cells · Tumor-infiltrating lymphocytes

Introduction

In tumor microenvironment, lymphocytes predominate in mononuclear infiltrates and represent an adaptive antitumor immune response. The most abundant population of tumor-associated lymphocytes is T cells, which include many subpopulations differing in their function. Among them, CD8+ cytotoxic T lymphocytes (CTLs) are particularly known for their cytolytic activity against cancer cells. On the contrary, GATA3+ T helper 2 (Th2) cells and FOXP3+ regulatory T lymphocytes (Tregs) downregulate antitumor immune response by impairing antigen presentation, activity, and cytotoxicity of other immune cells, thus promoting tumor growth and immune tolerance. Tregs originate from naïve T cells both in the thymus and at the periphery, and the process of their differentiation is orchestrated by a specific cytokine milieu. Molecules secreted by tumor-infiltrating lymphocytes (TILs), cancer cells, and other components of tumor microenvironment affect the composition and function of the cancer milieu, and, thereby, modulate the course of breast cancer progression [1–6]. It was observed that TILs rich in Tregs interact with cancer-associated fibroblasts, contributing to stromal remodeling, that presumably promote tumor growth and invasion [7]. The density of T cells was reported to increase as mammary tumor progresses from normal breast tissue, through benign and in situ lesions, to invasive ductal

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✉ Krzysztof Okoń
k.okon@uj.edu.pl

[1] Department of Pathomorphology, Jagiellonian University Medical College, ul. Grzegórzecka 16, 31-531 Kraków, Poland

[2] Department of General, Oncological, and Gastrointestinal Surgery, Jagiellonian University Medical College, Kraków, Poland

[3] Department of Internal Medicine, Jagiellonian University Medical College, Kraków, Poland

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cancers; this finding was interpreted as a stepwise increase in immunity with the course of mammary tumorigenesis [5–8]. An antitumoral immunity shows plasticity (immunoediting) and changes in time from tumor elimination (based highly on CTLs), through the equilibrium phase to immune escape (characterized by immunosuppressive profile of tumor microenvironment and generation of Tregs). The process of immunoediting is thought to result from the shifted balance between respective T cell phenotypes and selection of non-immunogenic clones [9, 10]. In line with these, the immune response appears to be dysfunctional and skewed toward suppression in invasive breast tumor tissue [2, 11].

Since the discovery of the intrinsic molecular subtypes that differ in their genetic pattern and clinical aggressiveness, invasive breast cancer has become regarded as a heterogeneous disease [12, 13]. To a certain extent, the interplay between malignant breast tumor and TILs is dependent on tumor genetics and biology [10]. Moreover, there is growing evidence that prognostic and predictive relevance of TILs varies in breast cancer of different intrinsic subtypes [1, 4, 5, 10, 11]. Nonetheless, the relationships between composition of lymphocytic milieus and breast cancer molecular subtypes have not been fully elucidated so far. Relationships between cancer and its microenvironment are of great interest, as some chemotherapeutic agents may elicit or enhance antitumor immune reactions, and innate, adaptive, cellular, and humoral pathways may be involved in cancer cell killing [14]. Simultaneously, new therapeutic approaches that aim at inducing potent immune response are sought. This includes an increase of tumor immunogenicity, inhibition of immune evasion [10, 11], and enhancement of cytotoxic and Th1 response, as well as a reduction of regulatory and Th2 cell impact on neoplastic breast tissue [5].

In our study, we investigated the lymphocyte infiltrate composition in order to assess its relationships with invasive breast cancer molecular subtypes and the occurrence of other prognostic and predictive markers for this disease. For this purpose, we evaluated both numbers of CTLs, Tregs, and Th2 cells and their percentages in tumor-associated immune infiltrates. Moreover, we also calculated proportions of investigated cells to assess differences in their relative quantities with regard to clinico-pathological indicators in breast cancer.

Material and methods

Material

The material comprised 106 routinely processed, formalin-fixed paraffin-embedded tissues of primary invasive breast carcinomas diagnosed between 2002 and 2015. The patients who received presurgical chemotherapy were excluded from the study. The archival hematoxylin-eosin–stained slides were re-evaluated and representative, well-preserved specimens were chosen for immunohistochemistry. The Nottingham Histologic Grade system was used for grading, and the 8th edition of the AJCC system was used for staging [15].

Immunohistochemistry

Immunohistochemistry (IHC) for CD8, FOXP3, GATA3, estrogen receptor (ER), progesterone receptor (PR), and Ki67 protein was performed according to the protocol routinely used in our laboratory. The selected blocks were cut into 4-μm-thick sections. Antigen retrieval was performed by incubating the slides in a citrate buffer (pH 6.0; 0.01 M) or EDTA (pH 8.0; 0.01 M) at 97 °C in a water bath for 40 and 30 min, respectively. The UltraVision Quanto Detection system (Lab Vision, Thermo Fisher Scientific, USA) and 3,3′-diaminobenzidine as chromogen were used, and the slides were counterstained with Mayer hematoxylin (Thermo Fisher Scientific, Waltham, USA) and coverslipped. Immunohistochemistry for HER2 (PATHWAY 4B5, Ventana Medical Systems Inc., USA) was performed on a BenchMark BMK Classic autostainer (Ventana, USA) using an UltraView DAB Detection Kit (Ventana Medical Systems Inc., USA). The primary antibodies used are listed in Table 1.

For specimens with HER2 status 2+ in immunohistochemistry, fluorescence in situ hybridization (FISH) was conducted. FISH was performed using a PathVysion HER-2 DNA Probe Kit II (Abbott Molecular, USA) according to the manufacturer's protocol. The red Locus Specific Identifier (LSI) HER-2/neu and green Centromere Enumeration Probe (CEP 17) signals were counted on a fluorescence microscope equipped with specific filter sets and HER-2/neu to CEP17 ratio > 2.0 was considered as HER2/neu amplification [16].

Evaluation of immunostaining and lymphocytic infiltrates

The immunostained slides were initially scanned on a Nikon Labophot-2 optical microscope (Tokyo, Japan) at low magnification (× 100), and the areas with the highest number of positive cells were chosen. Then, for CD8+ and FOXP3+ T cell populations, positively stained cells were counted in 5 high-power fields (HPFs; × 400, 0.2-mm^2 field area) and added together, which represented cell counts in 1 mm^2 of the examined tissue. The positive cells located in tumor-surrounding stroma, no further than 1 HPF from the tumor edge, were regarded as invasive margin or tumor edge, while positive cells located within neoplastic tissue (i.e., in contact with cancer cells) were considered intratumoral or intraepithelial population (Fig. 1). Additionally, for CD8+, FOXP3+, and GATA3+ cells, the percentages of positively stained cells were

Table 1 Antibodies used in the study

	Clone	Dilution	Antigen retrieval	Incubation time	Manufacturer
CD8	C8/144B	1:100	Citrate	60 min	Dako, USA
FOXP3	236A/E7	1:100	EDTA	30 min	Abcam, UK
GATA 3	L50–823	1:100	EDTA	30 min	Cell Marque, USA
ER	6F11	1:100	Citrate	30 min	Novocastra (Leica Biosystems, Germany)
PR	PgR636	1:100	Citrate	60 min	Dako, USA
Ki67	MIB-1	1:100	Citrate	30 min	Dako, USA

visually evaluated in mononuclear infiltrate at the invasion front. The percentages of investigated cells were evaluated in 5 HPFs and averaged. Finally, the ratios of examined T cell populations were calculated separately for their numbers in the intratumoral area and at the tumor edge, as well as for their percentages in tumor-surrounding stroma. In the study, CD8+, FOXP3+, and GATA3+ were considered CTLs, Tregs, and Th2 cells, respectively.

Additionally, evaluation of TILs was performed in tumor stroma, in the whole tissue section, according to the recommendations of the International TILs Working Group 2014 [17].

Positive ER and PR expression thresholds were set when ≥ 1% of neoplastic cells showed positive immunostaining. The threshold for discriminating between low and high Ki67 expression was set at ≥ 20% of positive cells. Scoring of the HER2 staining was performed by the standard method [16].

Definition of breast cancer molecular subtypes

The cases were classified into molecular subtypes according to the St Gallen 2015 International Expert Consensus [13]: luminal A (ER+ and PR ≥ 20%, Ki67 < 20%, HER2−), luminal B/HER2− (ER+, HER2− with PR <20% and/or Ki67 ≥ 20%), luminal B/HER2+ (ER+ or PR+, HER2+), HER2+ non-luminal (ER−/PR−/HER2+), and triple-negative breast cancer (ER−/PR−/HER2−).

Statistical analysis

To assess the differences between groups, the ANOVA Kruskal–Wallis and Mann–Whitney *U* tests were performed. A *t* test was applied for normally distributed variables. The correlations between groups were evaluated by using the Spearman rank test. All analyses were performed using Statistica 13 (StatSoft Inc., USA). In brackets, the data are expressed as mean values ± standard deviations; p values < 0.05 were considered statistically significant.

Results

A detailed description of the study group is shown in Table 2.

Lymphocyte infiltrate composition in different breast cancer molecular subtypes

We noted that both TNBC and HER2+ non-luminal tumors were more abundantly infiltrated by lymphocytes, as seen on H&E sections, in comparison with luminal A lesions ($p < 0.001$). Moreover, the HER2+ non-luminal subtype was also associated with a higher TIL level than lesions of luminal B phenotype ($p < 0.007$). With reference to the numbers of individual lymphocyte populations, we observed significantly more CTLs at the invasive margin of TNBC and HER2+ non-luminal cancers than in luminal A tumors ($p < 0.001$ for both

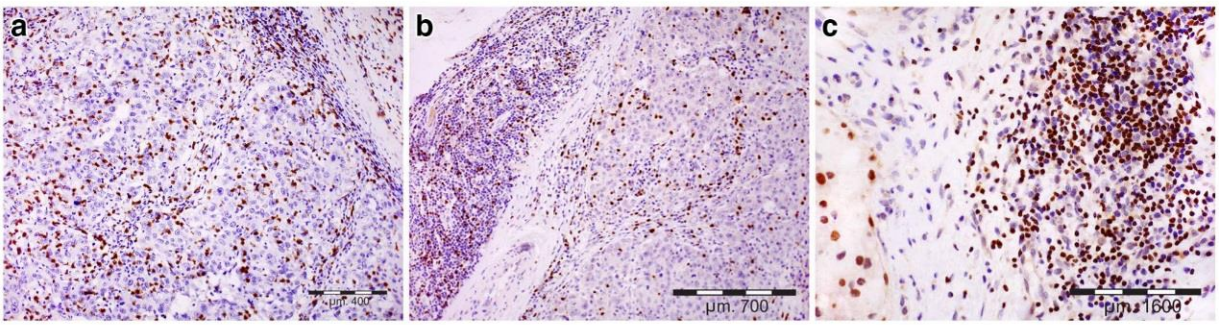

Fig. 1 Representative infiltration of T cell subpopulations in investigated invasive breast cancer tissues; **a** CTLs, **b** Tregs, **c** Th2 cell infiltrates (at invasive margin); immunostaining for CD8, FOXP3, and GATA3, respectively; light microscopy, magnification used: × 200 (**a**, **b**) and × 400 (**c**)

Table 2 Characteristics of the study group

Characteristic	No. of cases	Percentage
Total	106	100.0
Mean patient age 55.7 (range 29–87)		
Tumor size		
pT1	56	52.8
pT2	46	43.4
pT3	3	2.8
pT4	1	0.9
Nodal involvement		
pN0	53	50.0
pN1	32	30.2
pN2	8	7.5
pN3	12	11.3
Stage of disease		
I	40	37.7
II	42	39.6
III	22	20.7
IV	2	1.9
Nottingham histologic grade		
G1	16	15.1
G2	34	32.1
G3	56	52.8
Histological type		
NOS	93	87.7
Lobular	11	10.4
Other	2	1.9
Molecular subtype		
Luminal A	33	31.1
Luminal B	14	13.2
Luminal B/HER2+	12	11.3
HER2+ non-luminal	20	18.9
Triple negative	27	25.5

comparisons). The same observation was made for Tregs of the tumor edge (TNBC vs. luminal A: $p < 0.006$; HER2+ non-luminal vs luminal A: $p < 0.001$), and this population was more abundant in luminal B cancers, as compared with luminal A tumors ($p < 0.050$). The intratumoral Treg level was also significantly higher in TNBC than in luminal A tumors ($p < 0.003$). No statistically significant differences were observed for CTL/Treg number ratio both within neoplastic epithelium and at the invasive margin (Fig. 2, Tables 3 and 4). Regarding the percentages of analyzed T cell populations in tumor-associated infiltrates, we observed higher Treg/Th2 cell percentage ratio at the tumor edge of HER2+ non-luminal as compared with luminal A lesions ($p < 0.040$; Fig. 2, Table 4).

According to St Gallen 2015 distinction between luminal A and B molecular subtypes, the latter was characterized by higher numbers of CTLs (723.2 ± 406.4 vs. 445.8 ± 303.6, $p < 0.025$) and Tregs (252.2 ± 140.9 vs. 127.0 ± 110.8, $p < 0.005$) located at the invasive margin of a tumor.

As far as the HER2 status was concerned, we found that more abundant TILs as well as more numerous CTLs and Tregs at the invasion front were associated with HER2 overexpression ($p < 0.001$, $p < 0.010$, and $p < 0.001$, respectively; Tables 3 and 4).

The expression of hormone receptors showed a negative correlation with CTL counts at the tumor edge (ER: $R = -0.46$, PR: -0.47, $p < 0.001$), intratumoral and invasive margin Treg numbers (ER: $R = -0.26$, $p < 0.008$ and $R = -0.44$, $p < 0.001$; PR: $R = -0.26$, $p < 0.007$ and $R = -0.45$, $p < 0.001$ respectively), the percentage of Tregs at tumor edge (ER: not significant, PR: $R = -0.21$, $p < 0.035$), TIL level (ER: $R = -0.57$, PR: $R = -0.54$, $p < 0.001$), and the Treg/Th2 cell percentage ratio (ER: $R = -0.34$, $p < 0.002$; PR: $R = -0.31$ $p < 0.001$) as well as a positive correlation with both Th2 cell percentage (ER: $R = 0.25$, $p < 0.015$; PR: $R = 0.19$, $p < 0.050$) and CTL/Th2 cell percentage ratio (ER: $R = 0.21$, $p < 0.035$; PR: $R = 0.22$, $p < 0.030$) at tumor edge. Expression of Ki67 in neoplastic cells correlated positively with CTL and Treg counts both within tumor and at the invasive margin (CTLs: $R = 0.28$, $p < 0.005$ and $R = 0.41$, $p < 0.001$; Tregs: $R = 0.42$ and $R = 0.38$, $p < 0.001$, respectively), Treg percentage at invasion front ($R = 0.27$, $p < 0.007$), TIL infiltrate ($R = 0.46$, $p < 0.001$), and Treg/Th2 cell percentage ratio ($R = 0.29$, $p < 0.004$).

The relationships between lymphocyte infiltrate composition and other prognostic indicators in breast cancer

With regard to the tumor size, we stratified the analyzed samples into small tumors (pT1) and the lesions of diameter greater than 2 cm (pT > 1). We observed that the latter was characterized by more abundant TIL infiltrates (33.6 ± 20.7 vs. 23.8 ± 18.2, $p < 0.007$), higher intratumoral CTL number (172.7 ± 146.8 vs. 131.3 ± 179.9, $p < 0.007$), and higher Treg/Th2 cell percentage ratio at the invasion front (0.25 ± 0.12 vs. 0.22 ± 0.18, $p < 0.045$). When cancers of different phenotypes were analyzed separately, in cases of luminal cancers, a greater tumor diameter was associated with more numerous intratumoral CTLs (90.2 ± 67.0 vs. 170.1 ± 123.0, $p < 0.006$) and Tregs (35.9 ± 39.8 vs. 66.5 ± 57.9, $p < 0.010$), higher Treg percentage at the tumor edge (8.9 ± 4.1 vs. 11.2 ± 4.1, $p < 0.020$), TIL level (16.4 ± 14.8 vs. 26.4 ± 18.4, $p < 0.025$), and Treg/Th2 cell percentage ratio at invasive margin (0.18 ± 0.12 vs. 0.23 ± 0.09, $p < 0.015$). No significant differences were observed between the composition of lymphocytic infiltrate and tumor size in the non-luminal group.

Primary breast cancers that developed nodal metastases showed more prominent TILs (32.9 ± 19.3 vs. 24.6 ± 19.7,

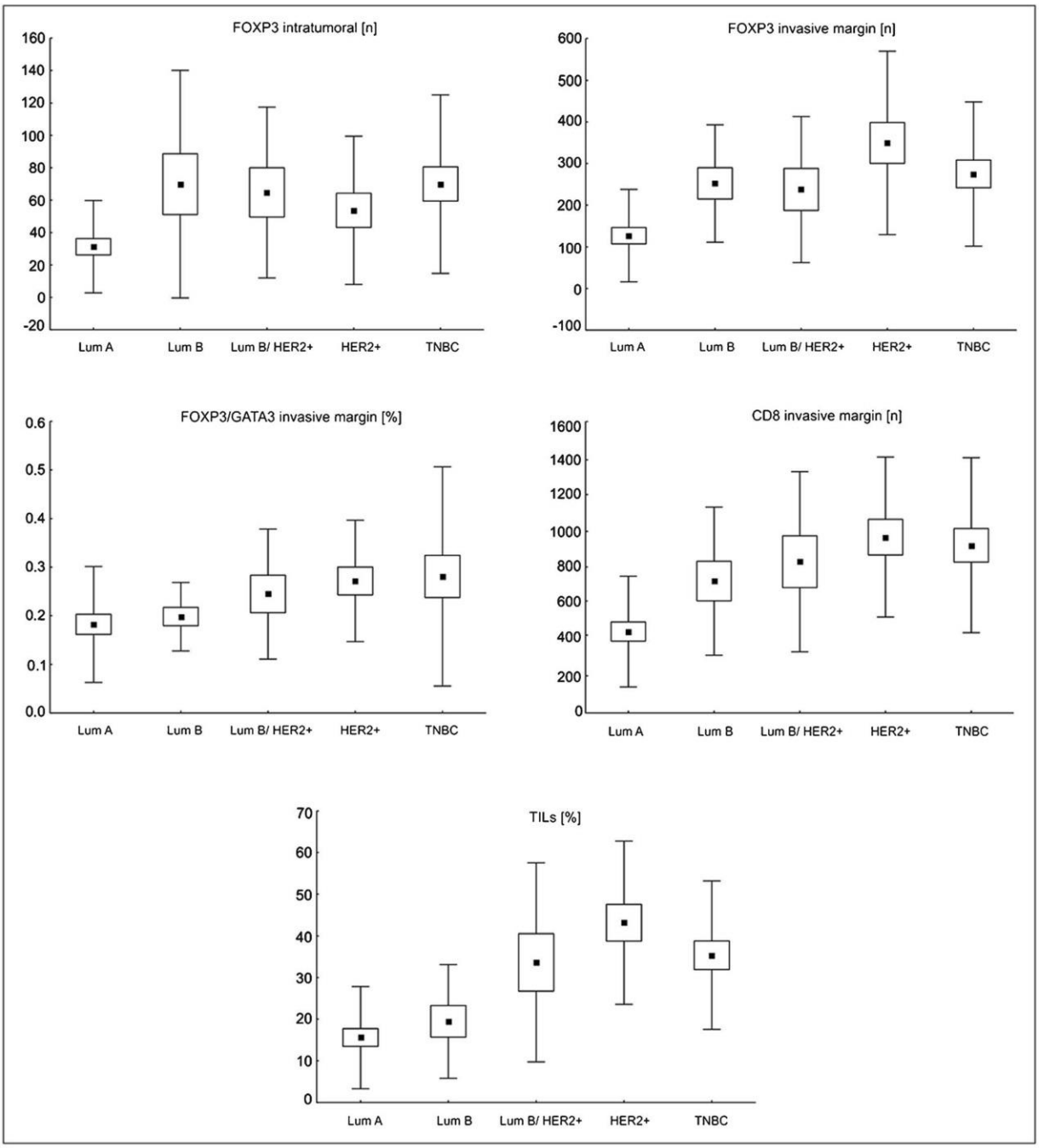

Fig. 2 The significant differences in T cell subpopulation infiltrates of primary breast cancer tissue with reference to molecular subtypes. Lum A, luminal A; Lum B, luminal B/ HER2−; Lum B/HER2+, luminal B/HER2+; HER2+, HER2+ non-luminal; TNBC, triple-negative subtype. Central point is the arithmetic mean, box is the arithmetic mean ± standard error, and whisker is the arithmetic mean ± standard deviation.

$p < 0.015$), higher Treg percentage (11.2 ± 4.2 vs. 9.4 ± 3.7, $p < 0.020$), and Treg/Th2 cell percentage ratio at the invasion front (0.25 ± 0.13 vs. 0.21 ± 0.18, $p < 0.030$) in comparison with metastasis-free cases. After stratification into luminal and non-luminal cancers, we noted that a higher intratumoral CTL/Treg number ratio was associated with regional lymph

Table 3 Associations between quantities of investigated T cell populations and selected prognostic factors in breast cancers

	Intratumoral						Invasive edge stroma					
	CD8 (n)			FOXP3 (n)			CD8 (n)			FOXP3 (n)		
	Mean	SD	p	Mean	SD	p	Mean	SD	p	Mean	SD	p
St Gallen 2015 molecular subtype												
Luminal A	106.1	102.4	NS	31.3	28.5	< 0.005	445.9	303.6	< 0.001	127.0	110.8	< 0.001
Luminal B	141.1	105.1		69.9	70.3		723.2	406.4		252.2	140.9	
Luminal B/HER2+	133.4	82.1		64.7	52.7		829.5	493.6		237.7	175.3	
HER2+ non-luminal	126.5	132.6		53.7	45.7		964.4	439.1		349.4	219.9	
TNBC	234.6	257.0		70.0	55.1		919.5	480.2		275.0	173.1	
Histological grade												
G1	62.3	34.7	< 0.002	26.6	26.1	< 0.001	420.4	259.4	< 0.001	112.7	83.2	< 0.001
G2	119.5	107.1		43.0	41.2		571.9	367.9		176.9	129.8	
G3	193.2	200.5		68.7	56.2		941.7	465.5		307.5	194.0	
HER2 overexpression												
No	159.6	183.1	NS	53.0	52.1	NS	671.1	445.2	< 0.010	205.7	157.1	< 0.008
Yes	129.2	114.2		58.0	48.0		913.8	457.2		307.5	208.7	

	CD8/FOXP3		
	Mean	SD	p
St Gallen 2015 molecular subtype			
Luminal A	5.29	3.74	NS
Luminal B	3.89	3.28	
Luminal B/HER2+	4.39	2.30	
HER2+ non-luminal	3.39	1.89	
TNBC	3.92	1.83	
Histological grade			
G1	5.11	3.62	NS
G2	4.63	3.32	
G3	3.85	2.20	
HER2 overexpression			
No	4.52	3.10	NS
Yes	3.77	2.08	

	CD8/FOXP3		
	Mean	SD	p
St Gallen 2015 molecular subtype			
Luminal A	4.62	3.52	NS
Luminal B	3.79	3.36	
Luminal B/HER2+	2.81	1.57	
HER2+ non-luminal	2.88	1.98	
TNBC	3.39	2.36	
Histological grade			
G1	2.95	1.31	NS
G2	4.69	3.96	
G3	3.25	2.12	
HER2 overexpression			
No	4.00	3.12	NS
Yes	2.85	1.81	

Kruskal–Wallis test was performed

NS not significant, *n* cell number counted

node metastases ($p < 0.035$) in the latter group. For luminal tumors, there was a tendency toward higher Treg/Th2 cell percentage ratio at the edge of tumors of positive nodal status ($p = 0.051$; Fig. 3).

In terms of tumor histologic grade, we observed that tumor-associated infiltrates differed in numbers of CTLs in intratumoral area and tumor edge ($p < 0.002$ and $p < 0.001$, respectively) as well as in both populations of Tregs (intratumoral $p < 0.003$, invasive margin $p < 0.001$) and TIL abundance ($p < 0.001$) as well as in the percentage of Treg lymphocytes ($p < 0.008$) and the Treg/Th2 cell percentage ratio at the invasion front ($p < 0.035$), with their increased levels in G3 as compared to G1 tumors. Additionally, for Treg numbers, both in intratumoral and invasive margin location, CTL numbers at the tumor edge, and TIL infiltrates, significantly higher levels were noted in G3 than in G2 cancers ($p < 0.040$, $p < 0.006$, $p < 0.001$, and $p < 0.001$, respectively; Tables 3 and 4).

As far as the histologic type was concerned, NOS cancers were characterized by more abundant TILs (30.3 ± 20.3 vs. 13.1 ± 9.3, $p < 0.003$), more numerous CTLs of invasive margin (793.1 ± 461.7 vs. 380.0 ± 286.1, $p < 0.003$) and Tregs at the tumor edge (256.4 ± 180.8 vs. 101.0 ± 96.2, $p < 0.002$), and a higher Treg percentage at invasion front (10.6 ± 4.1 vs. 8.6 ± 3.9, $p < 0.050$) as well as decreased intraepithelial CTL/Treg number ratio (3.27 ± 2.43 vs. 7.09 ± 3.86, $p < 0.001$) as compared with CLI lesions.

The tumor tissue excisions obtained from stage I patients were related to a significantly lower TIL level (19.7 ± 15.1 vs. 36.1 ± 22.5, $p < 0.001$), lower counts of CTLs (596.8 ± 449.8 vs. 864.3 ± 472.1, $p < 0.015$), and Tregs at invasive margin (176.3 ± 152.8 vs. 288.7 ± 206.7, $p < 0.015$) as well as a lower Treg percentage (9.0 ± 4.1 vs. 10.5 ± 2.8, $p < 0.045$) and Treg/Th2 cell percentage ratio at the tumor edge (0.20 ± 0.20 vs. 0.24 ± 0.12, $p < 0.035$) in comparison with stage II cancers; for the two latter parameters, such a difference was also observed between stage I and stage III/IV cancers (stage III/IV: Treg percentage -12.3 ± 5.0, $p < 0.009$, Treg/Th2 cell percentage ratio -0.26 ± 0.12, $p < 0.020$).

Discussion

CTLs are commonly considered as a part of cancer immune surveillance. Some research into breast cancer-linked CTLs revealed their lytic and proapoptotic activity [18] as well as a memory phenotype in the majority of this cell population, particularly when high-grade lesions were concerned [19]. Tsang et al. [20] observed that CD8+ and FOXP3+ expressions were mutually exclusive in double immunohistochemical staining of breast cancer microenvironment. On the contrary, the existence of fractions of CTLs that express FOXP3 secrete immunosuppressive interleukin (IL)-10 [21] and co-express molecules associated with anergy, exhaustion, or

Table 4 Associations between the percentage of analyzed cells in stromal lymphocytic infiltrates at the invasive margin and selected prognostic factors in breast cancer

	CD8 (%)			FOXP3 (%)			GATA3 (%)			CD8/FOXP3			CD8/GATA3			FOXP3/GATA3			TILs (%)		
	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*
St Gallen 2015 molecular subtype																					
Luminal A	33.2	11.8	NS	9.1	4.5	NS	52.5	11.5	NS	4.86	3.80	NS	0.64	0.21	NS	0.18	0.12	< 0.025	15.6	12.2	< 0.001
Luminal B	35.9	11.5		10.4	1.8		55.4	11.9		3.52	1.08		0.65	0.18		0.20	0.07		19.5	13.6	
Luminal B/HER2+	43.8	26.6		11.0	5.2		49.0	15.1		4.93	4.82		0.93	0.48		0.24	0.13		33.6	23.9	
HER2+ non-luminal	34.1	12.3		12.1	4.9		49.0	12.2		3.18	1.46		0.75	0.35		0.27	0.12		43.1	19.6	
TNBC	32.8	11.0		10.2	2.6		45.4	16.0		3.46	1.70		0.89	0.74		0.28	0.22		35.3	17.8	
Histological grade																					
G1	30.3	12.6	NS	8.3	4.9	< 0.015	50.8	14.5	NS	5.26	4.70	NS	0.61	0.23	NS	0.19	0.14	< 0.035	14.1	12.5	< 0.001
G2	34.1	11.9		10.5	4.5		52.6	11.7		3.77	2.14		0.66	0.22		0.20	0.10		19.5	12.3	
G3	36.6	15.8		10.8	3.4		48.3	14.3		3.79	2.64		0.86	0.59		0.26	0.18		37.8	20.6	
HER2 overexpression																					
No	33.6	11.4	NS	9.7	3.4	NS	50.5	13.8	NS	4.08	2.82	NS	0.73	0.49	NS	0.22	0.16	NS	23.6	17.2	< 0.001
Yes	37.8	19.2		11.7	4.9		49.0	13.2		3.84	3.21		0.82	0.41		0.26	0.13		39.6	21.4	

Kruskal–Wallis test was performed

NS not significant, % cell percentage evaluated

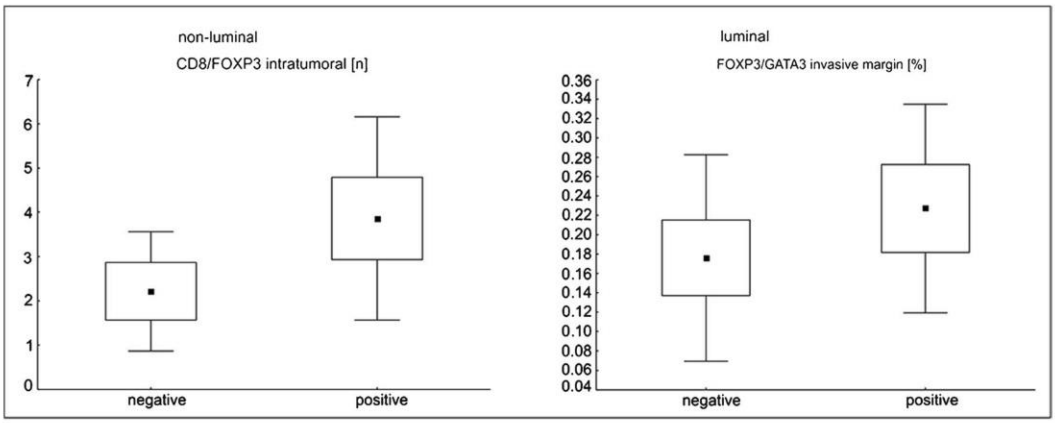

Fig. 3 The significant differences in T cell subpopulation infiltrate of primary breast cancer tissue with reference to nodal status, after stratification upon breast cancer phenotype. Central point is the arithmetic mean, box is the arithmetic mean ± 2*standard error, and whisker is the arithmetic mean ± 0.95*standard deviation.

unresponsiveness in tumor-infiltrating lymphocytes [22] was reported.

The data on associations between lymphocytic infiltrate and breast cancer molecular subtype are inconclusive. In line with our study, some authors reported that increased CTL counts were associated with ER and PR negativity, HER2 overexpression [23–25], and higher Ki67 level [25, 26] in breast cancer, while the results from other publications question these findings [20, 26, 27]. Liu et al. hypothesized that the location of immune infiltrates (intraepithelial or peritumoral) may influence activation of its cells, as cell populations within a tumor are dispersed and their interactions are impeded [23]. In the Miyan et al. study, a significantly increased number of CTLs was observed at the invasive margin of basal-like and luminal B/HER2+, with their lowest counts in luminal A lesions [25]; however, the authors applied St Gallen 2013 molecular subtype classification in their research. Tsang et al. suggested that the mechanism of intratumoral recruitment and survival of lymphocytes may differ between subtypes, and that in HER2-positive tumors, CTL migration is preferred over Treg influx [20]. Our observation of high CTL counts in HER2+ non-luminal and triple-negative phenotypes was made for a population of these cells located at tumor edge, exclusively. On the other hand, the percentages of CTLs in tumor-associated infiltrates at invasive margin did not differ between subtypes. Therefore, the increased numbers of CTLs may reflect more abundant TILs noted in these breast cancer subtypes rather than a shift in immune response toward more potent cell killing. A favorable prognostic value of high levels of breast cancer-related CTLs was attributed to ER-negative as well as ER+/HER2+ phenotypes [28]. Moreover, high FAS protein expression in ER-negative cancers was proposed to be one of the contributing factors to beneficial impact of CTL on patient survival. Of note, their adverse effect on ER-positive FAS-high patient outcome indicated different functions of tumor-infiltrating CTLs with respect to breast cancer subtypes [29].

Literature data on relationships between CTLs and other prognostic factors in invasive breast cancer are also ambiguous. Some authors [14, 19, 23–25], but not all [23, 27, 30], report higher CTL counts in tumors of higher histological grade and size, which is in line with our findings. We noted that relationships between more numerous CTL infiltrates and higher grades concerned both intraepithelial area and tumor edge, while tumors of greater diameter were characterized only by a more abundant intraepithelial population. After stratification, the latter finding remained significant for luminal lesions, exclusively. Regarding lymph node metastases, higher counts [26, 27, 31] as well as a higher frequency of CTLs [19] were observed in primary tumors with nodal spread, but such association was not observed by other groups [23, 32]. In our study, the CTL infiltrates did not differ according to nodal status.

FOXP3 is a transcription marker expressed in a vast majority of breast cancer-infiltrating Tregs [33]. Similar to CTLs, Tregs were observed to accumulate in breast tumor and its immediate milieu, in comparison with normal tissue. Moreover, tumor-infiltrating Tregs were more frequently characterized by activated, strongly immunosuppressive but exhausted phenotype, which may correspond with immune tolerance [22]. As far as the breast cancer intrinsic subtypes were concerned, the most numerous regulatory T cell infiltrate is frequently associated with either TNBC or HER2+ non-luminal phenotype of tumors, while the lowest Treg numbers are observed in luminal A lesions [23, 25, 34], which is in accordance with our results. Moreover, the stronger Treg infiltrate of TNBC concerns both the surrounding stroma and tumor center. Such findings indicated associations between tumor biological features and immunological response in invasive breast cancers [25] as well as more immunosuppressive

microenvironment of clinically aggressive subtypes [23]. Moreover, the increased proportion of Tregs in a lymphocytic milieu [35], as well as their higher quantities, was noted in TNBC and hormone receptor (HR)-negative and HER2-overexpressed breast tumors [23, 34], which supports our results. We also observed slight correlations between Treg percentage in immune cell infiltrates of invasive edge and either a drop of PR or an increase in Ki67 expression that suggest a shift toward a more immunotolerant milieu in PR-negative or intensively proliferating breast cancers. More abundant Tregs were found in the center of ER-positive and in the peritumoral area of ER−/HER2+ cancers, while a lower number of cells infiltrated the intratumoral site of ER−/HER2+ tumors in Tsang et al. research [20]. Some authors did not observe any differences in Treg infiltrates regarding breast cancer intrinsic subtypes [36]. In ER-negative tumors, more intensive Treg infiltration was associated with better disease-free survival [33]. Similar to CTLs, higher levels of Tregs were often observed in high-grade cancers [14, 23, 35], but their relationship with tumor size and nodal status is controversial [22, 23, 26, 34]. We found increased Treg numbers and percentages in luminal breast tumors of greater size, as well as a higher proportion of Tregs in the microenvironment of node-positive invasive tumors, that suggests a regulatory bias in TILs of more advanced cancers.

The ratio of CTL to Treg numbers is regarded as an indicator of cytotoxicity. A higher CD8+/FOXP3+ ratio was observed by Liu et al. in the peritumoral area of non-luminal breast cancers and indicated greater cytolytic potential of the lymphocytic milieu surrounding these tumors [23]. It was postulated that the change of this parameter is rather due to Treg reduction than CTL recruitment [14]. Complementary FOXP3+/CD8+ cell ratio was suggested to reflect an immune evasion of a tumor, with its higher values in tumor center as compared with the peritumoral area. In breast tumor, it was associated with ER negativity, higher proliferation rate, and high histological grade, but not with tumor size or nodal involvement [25]. In our study, increased intraepithelial CTL/Treg number ratio was associated with lobular histology and metastatic disease of non-luminal cancers. Thus, we hypothesize that, for breast tumors of non-luminal phenotype, their spread is associated with cytotoxicity failure.

Information on tumor-associated Th2 cells in breast cancer is scarce. Th2 was reported as a predominant population of T helper cells in a mouse model of luminal breast cancer; their lower counts in the tumor milieu were associated with decreased pulmonary metastasis by Zhang et al. [37]. Moreover, higher levels of Th2 cytokines—IL-10 [14] and IL-5 [3]—were related to the lack of pathologic complete response after chemotherapy and worse survival in breast cancer patients, respectively. In our study, tumor-associated Th2 cells showed a slight correlation with HR. On the contrary, the expression of genes related to Th2 signaling was more prominent in basal cancers by Kristensen et al. [38]. Ghirelli et al. showed that cytokines secreted by breast cancer tissue resulted in regulatory Th2 bias of tumor-related immunity, which supposedly was GATA3 independent [39]. To assess immunoregulatory and suppressive potential of immune infiltrates, we evaluated the Treg/Th2 cell percentage ratio. Its high value was associated with adverse prognostic indicators: HER2+ non-luminal subtype, decrease in HR expression, increasing proliferation rate, and higher grade as well as greater tumor size and positive lymph node status, particularly in luminal cancers. In addition, the higher percentage ratio of CTLs to Th2 at invasion front modestly correlated with HR expression. Thus, we hypothesize that the bias of immunosuppressive microenvironment toward regulatory function is associated with clinically more aggressive breast cancers.

Our recent research studies aimed at evaluation of tumor microenvironment in primary invasive breast cancer have shown associations between higher quantities of mast cells and beneficial prognostic indicators [40] and relationships between T cell, B cell, and NK cell infiltrates and adverse clinical factors [41]. These findings induced us to presently investigate infiltrates of several T cell subpopulations in this disease. In conclusion, we observed that T cell infiltrates of primary invasive breast tumors differ in numbers and percentages of its individual populations regarding cancer molecular features and prognostic markers. Moreover, the relationships between lymphocytic composition and pT or nodal status vary according to cancer phenotype, suggesting that distinct mechanisms govern cancer progression in luminal and non-luminal lesions. As more numerous T cell subpopulations in breast cancer tissue may result from higher TIL levels, we additionally evaluated percentages of analyzed cells in tumor-surrounding infiltrates that should be resistant to the number of TILs. Thus, further investigation is needed to elucidate function of immune infiltrates in breast cancers of different molecular subtypes.

Author Contributions Anna Glajcar: assessment of the immunohistochemical slides; data analysis; drafting of the manuscript; preparation of the illustrations; preparation of the final text

Joanna Szpor: elaboration of the research concept; review of histological material; choice of cases for the study; general supervision of the study; drafting of the manuscript and preparation of the final text

Diana Hodorowicz-Zaniewska: provided clinical data; drafting of the manuscript and preparation of the final text

Katarzyna Ewa Tyrak: data analysis; preparation of the final text

Krzysztof Okoń: general supervision; elaboration of the research concept; elaboration of methodology; data analysis supervision; preparation of the final text

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Compliance with ethical standards

The study was approved by the Jagiellonian University Committee of Bioethics (consent number 122.6120.149.2016).

Conflict of interest The authors declare that they have no conflict of interest.

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Kraków, dnia 30.04.2019

mgr Anna Glajcar
.....
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment" (*Virchows Archiv*, 2017, 470: 505-515)

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

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Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje mój indywidualny wkład przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Anna Glajcar
.....
(podpis współautora)

Kraków, dnia 30.04.2019

Dr n. med. Joanna Szpor

.....

(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment" (*Virchows Archiv*, 2017, 470: 505-515)

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Joanna Szpor

(podpis współautora)

Kraków, dnia 08.02.2019

Lek. Agnieszka Marek

(tytuł zawodowy, imię i nazwisko)

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Agnieszka Marek
LEKARZ
2950823
[signature]

(podpis współautora)

(Informuję, że [illegible] [illegible] [illegible] [illegible], [illegible] [illegible] [illegible] dn. 27.06.2017r.

[signature])

Kraków, dnia 7.05.2018

Lek. Katarzyna Ewa Tyrak
.....
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

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Katarzyna Tyrak
.....
(podpis współautora)

2018/11/11
(date and place)

Florence Chan
(professional title, first name, last name)

OŚWIADCZENIE/ DECLARATION

Jako współautor pracy pt. "The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment" (*Virchows Archiv*, 2017, 470: 505-515)

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

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As a co-author of publication "The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment" (*Virchows Archiv*, 2017, 470: 505-515)

I declare my own contribution to preparation and development of the research as well as its presentation in the form of publication as:

contribution to the manuscript editing

In parallel, I agree to submission of the work by MSc Anna Glajcar as a part of the doctoral dissertation in the form of published, thematically coherent publication cycle.

I declare that unassisted and separable part of the work indicates an individual input of MSc Anna Glajcar to developing concepts, analysis performing, evaluation and interpretation of the results.

(signature of co-author)

Kraków, dnia 02.05.2018 r.

Lek. Joanna Streb

.....

(tytuł zawodowy, imię i nazwisko)

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dr n. med. JOANNA STREB
specjalista onkologii klinicznej
specjalista medycyny rodzinnej
5172961 9806143?2

.....

(podpis współautora)

Kraków, dnia 30.04.2019 r.

Dr n. med. Diana Hodorowicz-Zaniewska
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Dr hab. n. med. Krzysztof Okoń, prof UJ
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Joanna Szpor

(podpis współautora)

Kraków, dnia 7.05.2019

Lek. Katarzyna Ewa Tyrak

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