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Profile transkrypcyjne związane z układem immunologicznym w kontekście rozsiewu raka piersi

Immune-related transcriptional landscape of breast cancer in the context of its dissemination

Praca przedstawiona
Radzie Dyscypliny Nauki Biologiczne Uniwersytetu Gdańskiego
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Promotor: prof. dr hab. Anna J. Żaczek

Zakład Onkologii Translacyjnej
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Spis treści

Wykaz prac wchodzących w skład rozprawy doktorskiej

Publikacja 1 [P1]

Popeda M, Stokowy T, Bednarz-Knoll N, Jurek A, Niemira M, Bielska A, Kretowski A, Kalinowski L, Szade J, Markiewicz A, Zaczek AJ

NF-kappa B Signaling-Related Signatures Are Connected with the Mesenchymal Phenotype of Circulating Tumor Cells in Non-Metastatic Breast Cancer

Cancers (Basel). 2019 Dec 6;11(12):1961. doi: 10.3390/cancers11121961

Praca oryginalna – IF: 6,126 ; liczba cytowań: 13 (Web of Science, stan na dzień 09-06-2022)

Publikacja 2 [P2]

Bednarz-Knoll N, **Popęda M**, Kryczka T, Kozakiewicz B, Pogoda K, Szade J, Markiewicz A, Strzemecki D, Kalinowski L, Skokowski J, Liu J, Żaczek AJ

Higher platelet counts correlate to tumour progression and can be induced by intratumoural stroma in non-metastatic breast carcinomas

Br J Cancer. 2022 Feb;126(3):464-471. doi: 10.1038/s41416-021-01647-9

Praca oryginalna – IF: 7,640 ; liczba cytowań: 1 (Web of Science, stan na dzień 09-06-2022)

Publikacja 3 [P3]

Markiewicz A, Topa J, **Popęda M**, Szade J, Skokowski J, Wełnicka-Jaśkiewicz M, Żaczek A

Activation of epithelial-mesenchymal transition process during breast cancer progression – the impact of molecular subtype and stromal composition

Acta Biochim Pol. 2021 Aug 25;68(3):385-392. doi: 10.18388/abp.2020_5719

Praca oryginalna – IF: 2,149 ; liczba cytowań: 0 (Web of Science, stan na dzień 09-06-2022)

Publikacja 4 [P4]

Popeda M, Markiewicz A, Stokowy T, Szade J, Niemira M, Kretowski A, Bednarz-Knoll N, Zaczek AJ

Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients

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Praca oryginalna – IF: 4,379 ; liczba cytowań: 3 (Web of Science, stan na dzień 09-06-2022)

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Badania przedstawione w powyższych publikacjach były częścią projektu SONATA BIS 6 „Udział mikrośrodowiska guza w regulacji fenotypu rozsianych komórek nowotworowych raka piersi – badania *in vitro*, *in vivo* i na materiale klinicznym" finansowanego przez Narodowe Centrum Nauki (2016/22/E/NZ4/00664, kierownik projektu: prof. dr hab. Anna J. Żaczek).

Dane transkryptomiczne wygenerowane w ramach projektu doktorskiego zostały zdeponowane w repozytorium NCBI GEO pod numerem dostępu GSE180186.

Streszczenie

Przerzuty są najczęstszą przyczyną śmierci wśród chorych na złośliwe nowotwory. Rozsiew nowotworu to złożony proces, w który zaangażowane są zarówno komórki guza, jak również prawidłowe komórki otaczającego go mikrośrodowiska, w tym komórki układu immunologicznego. W kontekście rozsiewu istotne są również bezpośrednie i pośrednie oddziaływania, w które komórki guza wchodzą z elementami układu krążenia, np. płytkami krwi, czy osoczem i przekazywanymi za jego pośrednictwem cytokinami. Prezentowana praca skupia się na najczęstszym nowotworze u kobiet – raku piersi oraz mechanizmach towarzyszących jego rozsiewowi. Lepsze poznanie molekularnych mechanizmów przerzutowania oraz towarzyszących mu interakcji guz-mikrośrodowisko jest niezbędne dla rozwoju skutecznych metod detekcji rozsiewu, oceny ryzyka nawrotu choroby, czy wreszcie jej leczenia.

Rozprawa doktorska składa się z cyklu czterech oryginalnych publikacji naukowych, których głównym tematem są zmiany profilu transkrypcyjnego raka piersi, obejmującego geny związane z układem immunologicznym – tzw. immuno-transkryptomu, w kontekście różnych aspektów rozsiewu choroby. Przedmiotem analizy był archiwalny materiał tkankowy chorych o zbadanym statusie zajęcia węzłów chłonnych oraz obecności krążących komórek nowotworowych, obejmujący guzy pierwotne oraz sparowane przerzuty do węzłów chłonnych. Immuno-transkryptom tkanek archiwalnych, oceniony za pomocą technologii nCounter (NanoString), uważanej za złoty standard analiz ekspresji genów w materiale tkankowym utrwalonym w formalinie i zatopionym w parafinie, został poddany kompleksowej analizie bioinformatycznej i statystycznej.

Cele szczegółowe rozprawy to: ustalenie profili immunotranskryptomicznych: [1] guzów pierwotnych w odniesieniu do fenotypu krążących komórek nowotworowych (mezenchymalny vs. epitelialny) w krwi obwodowej; [2] guzów pierwotnych w odniesieniu do liczby płytek krwi (górny kwartyl zakresu normy vs. pozostały zakres normy liczby płytek krwi); [3a] guzów pierwotnych, które nie wytworzyły przerzuty do węzłów chłonnych, [3b] guzów pierwotnych, które wytworzyły przerzuty do węzłów chłonnych, [3c] przerzut ów do węzłów chłonnych, w odniesieniu do fenotypu występujących w nich komórek nowotworowych (mezenchymalny vs. epitelialny); [4] guzów pierwotnych w odniesieniu do sparowanych przerzutów do węzłów chłonnych – rozsiew drogą limfatyczną.

Uzyskane wyniki wskazują na: [1] podwyższoną ekspresję genów związanych ze ścieżką sygnalizacyjną NF-kB w guzach pierwotnych rozsiewających krążące komórki nowotworowe o fenotypie mezenchymalnym; [2] zwiększoną infiltrację limfocytów T CD8+ oraz komórek tucznych w fazie spoczynku oraz podwyższoną ekspresję genów kodujących cytokiny, które potencjalnie mogą skutkować nasileniem trombopoezy, aktywacji płytek oraz ich pro-nowotworowej aktywności: IL17A (*IL17A*), MDC (*CCL22*) oraz MMP1 (*MMP1*) w guzach pierwotnych chorych z podwyższoną liczbą płytek krwi; [3a] obniżoną ekspresję genów związanych z reakcją zapalną w mezenchymalnych guzach pierwotnych, które nie wytworzyły przerzutów do węzłów chłonnych; [3b] podwyższoną aktywność, potencjalnie również zwiększoną infiltrację, komórek prezentujących antygen w mezenchymalnych guzach pierwotnych, które wytworzyły przerzuty do węzłów chłonnych; [3c] obniżoną ekspresję genów związanych z produkcją interferonu w mezenchymalnych przerzutach do węzłów chłonnych; [4] obniżoną ekspresję genów związanych z układem dopełniacza w przerzutach do węzłów chłonnych względem sparowanych guzów pierwotnych.

Badania dostarczyły nowych informacji na temat udziału układu immunologicznego w rozsiewie raka piersi. Szlak sygnalizacyjny NF-kB i układ dopełniacza stanowią potencjalnie kluczowe dla przebiegu rozsiewu punkty, stąd mogą stać się atrakcyjnym punktem uchwytu terapii celujących w proces formowania przerzutów. Podobnie warte uwagi wydają się zależne od podścieliska procesy stymulujące trombopoezę, które w sposób pośredni sprzyjają rozsiewowi nowotworu. Wnioski płynące z przeprowadzonych badań mogą dać podstawę do opracowania nowych rozwiązań terapeutycznych dedykowanych dla chorych na raka piersi.

Abstract

Metastatic disease remains the leading cause of cancer-related deaths. Metastasis is a multistep process that involves the action of both tumour cells, and the surrounding tumour microenvironment, which is composed of normal cells, including cells of the immune system. In addition, metastasis is facilitated by both direct and indirect interplay between cancer cells and components of the blood, e.g., platelets, plasma or plasma cytokines. The subject of the presented work is breast cancer – the most frequently diagnosed neoplasm among females, and its dissemination. Better understanding of molecular mechanisms of cancer metastasis and the involved crosstalk between the tumour and its microenvironment is crucial for development of new methods of its diagnosis, relapse risk assessment, and treatment.

My PhD thesis is a cycle of four original research papers describing the changes of immune-related transcriptome of breast cancer in the context of various aspects of its dissemination. The analyses were performed on archival tissue material, comprising primary tumours and lymph node metastases, of patients with known status of nodal involvement and circulating tumour cells. The immunotranscriptome of archival material, generated using nCounter technology (NanoString) – the golden standard gene expression approach for formalin fixed and paraffin embedded tissues, was subjected to thorough bioinformatic and statistical analysis.

The project aimed to determine immunotranscriptome profiles of: [1] primary tumours according to the phenotype of circulating tumour cells (mesenchymal vs. epithelial) present in blood; [2] primary tumours according to the platelet count (higher normal count, defined as above upper quartile of the normal range, vs. normal count – the remaining normal range); [3a] primary tumours that did not metastasise to lymph nodes according to their phenotype (mesenchymal vs. epithelial); [3b] primary tumours that metastasised to lymph nodes according to their phenotype (mesenchymal vs. epithelial); [3c] lymph node metastases according to their phenotype (mesenchymal vs. epithelial); [4] primary tumours vs. paired lymph node metastases – changes associated with lymphatic spread.

The obtained results indicate: [1] an increased expression of NF-kB signalling-related genes in primary tumours seeding mesenchymal circulating tumour cells; [2] an elevated infiltration of CD8+ T lymphocytes and resting mast cells and increased expression of genes coding for cytokines promoting thrombopoiesis, platelet activation and their pro-tumorigenic function – IL17A (*IL17A*), MDC (*CCL22*) and MMP1 (*MMP1*), in primary tumours of patients with elevated platelet count; [3a] a decreased expression of inflammatory response-related genes in mesenchymal primary tumours that did not metastasise to lymph nodes; [3b] an increased activity, plausibly also elevated infiltration, of antigen presenting cells in mesenchymal primary tumours that metastasised to lymph nodes; [3c] a decreased expression of genes involved in interferon production in mesenchymal lymph node metastases; [4] a reduced expression of complement system-related genes in lymph nodes compared to matched primary tumours.

My studies provided new insight into the role of immune system in breast cancer dissemination. NF-kB signalling and the complement system seem to be crucial for metastasis formation, thus appear to be potent targets for anti-metastatic treatment. The stroma-induced thrombopoiesis also merits further investigation as it seems to promote the dissemination.

The outcomes of my project may serve as a basis for development of novel therapeutic approaches dedicated for breast cancer patients.

Wykaz skrótów

APRIL (TNFSF13)	czynnik indukujący proliferację (ang. *a proliferation-inducing ligand*, *tumor necrosis factor ligand superfamily member 13*)
ATG10	gen *Autophagy Related 10*
C2	gen *Complement C2*
C3	białko C3 układu dopełniacza (ang. *complement component 3*)
CAFs	fibroblasty związane z nowotworem (ang. *cancer-associated fibroblasts*)
CD63	gen *CD63 Molecule*
CD84	gen *CD84 Molecule*
CD164	gen *CD164 Molecule*
CIBERSORTx	algorytm cytometrii cyfrowej (ang. *Cell type Identification By Estimating Relative Subsets Of known RNA Transcripts – next generation*)
CK19	gen *Cytokeratin 19*
CTC	krążąca komórka nowotworowa (ang. *circulating tumour cell*)
CXCL3	gen *C-X-C Motif Chemokine Ligand 3*
CYLD	gen *CYLD Lysine 63 Deubiquitinase*
DAVID	serwer do funkcjonalnej analizy wzbogaceń oraz analizy adnotacji funkcjonalnych
DEGs	geny o zróżnicowanej ekspresji (ang. *differentially expressed genes*)
DFS	czas przeżycia wolnego od choroby (ang. *disease-free survival*)
DNA	kwas deoksyrybonukleinowy (ang. *deoxyribonucleic acid*)
E-cad	E-kadheryna (ang. *E-cadherin*)
ECM	macierz zewnątrzkomórkowa (ang. *extracellular matrix*)
EMT	przejście epitelialno-mezenchymalne (ang. *epithelial-mesenchymal transition*)
ENA78 (CXCL5)	białko nabłonkowe aktywujące neutrofile (ang. *epithelial-derived neutrophil-activating factor-78*, *C-X-C motif chemokine ligand 5*)
ER	receptor estrogenowy (ang. *estrogen receptor*)
FC	krotność zmiany (ang. *fold change*)
FFPE	utrwalony w formalinie i zatopiony w parafinie (ang. *formalin-fixed, paraffin-embedded*)
GATA3	gen *GATA Binding Protein 3*
GTex	projekt *Genotype-Tissue Expression*
HER2	receptor dla naskórkopochodnego czynnika wzrostu typu 2 (ang. *human epidermal growth factor receptor 2*)
HGF	czynnik wzrostu hepatocytów (ang. *hepatocyte growth factor*)
HLA-DQA1	gen *Major Histocompatibility Complex, Class II, DQ Alpha 1*
HLA-DQB1	gen *Major Histocompatibility Complex, Class II, DQ Beta 1*
hPC	górny kwartyl zakresu normy liczby płytek krwi w krwioobiegu, powyżej 289 000/µl (ang. *high normal platelet count*)
HR	hazard względny (ang. *hazard ratio*)

IFITM2	gen *Interferon Induced Transmembrane Protein 2*
IFNA7	gen *Interferon Alpha 7*
IFNAR1	gen *Interferon Alpha And Beta Receptor Subunit 1*
IL16	interleukina 16 (ang. *interleukin-16*)
IL17A	interleukina 17A (ang. *interleukin-17A*)
ITGA2B	gen *Integrin Subunit Alpha 2b*
LNM	przerzut do węzła chłonnego (ang. *lymph node metastasis*)
MCP3	białko chemotaktyczne monocytów 3 (ang. monocyte chemotactic protein-3)
MDC (CCL22)	chemokina wydzielana przez makrofagi (ang. *macrophage-derived chemokine, C-C motif chemokine 22*)
MERTK	gen *MER Proto-Oncogene, Tyrosine Kinase*
MET	przejście mezenchymalno-epitelialne (ang. *mesenchymal-epithelial transition*)
MMP1	metaloproteinaza macierzy pozakomórkowej 1 (ang. *matrix metalloproteinase-1*)
N-	węzły chłonne wolne od przerzutów raka
N+	węzły chłonne zajęte przez przerzut raka
N-cad	N-kadheryna (ang. *N-cadherin*)
NCBI GEO	baza *Gene Expression Omnibus* w ramach serwera *National Center for Biotechnology Information*
NF-kB	czynnik transkrypcyjny NF kappa B (ang. *nuclear factor kappa-light-chain-enhancer of activated B cells*)
nPC	zakres normy liczby płytek w krwioobiegu poniżej górnego kwartyla, 289 000/µl (ang. *normal platelet count*)
NST	rak naciekający bez specjalnego typu (ang. *invasive carcinoma of no special type*)
OS	czas przeżycia całkowitego (ang. *overall survival*)
PgR	receptor progesteronowy (ang. *progesteron receptor*)
PRG2	gen *Proteoglycan 2, Pro Eosinophil Major Basic Protein*
PSMD7	gen *Proteasome 26S Subunit, Non-ATPase 7*
PT	guz pierwotny (ang. *primary tumour*)
PTGS2	gen *Prostaglandin-Endoperoxide Synthase 2*
RNA	kwas rybonukleinowy (ang. *ribonucleic acid*)
RNA-seq	sekwencjowanie RNA (ang. *RNA sequencing*)
S100B	gen *S100 Calcium Binding Protein B*
SCF	czynnik wzrostu komórek macierzystych (ang. *stem cell factor*)
SIP1 (ZEB2)	gen *Zinc Finger E-Box Binding Homeobox 2*
SLUG (SNAI2)	gen *Snail Family Transcriptional Repressor 2*
TCGA	projekt *The Cancer Genome Atlas*
TNM	klasyfikacja służąca do określania stopnia zaawansowania klinicznego nowotworu (ang. *T – tumour*, *N – node*, *M – metastasis*)
TWIST1	gen *Twist Family BHLH Transcription Factor 1*
VIM	wimentyna (ang. *vimentin*)

Wprowadzenie

Obecnie rak piersi jest najczęściej diagnozowanym nowotworem u kobiet, jak również najczęstszą przyczyną śmierci u kobiet chorych na nowotwory na świecie [1]. W Polsce pozostaje najczęściej diagnozowanym nowotworem (19 620 z 85 659 – 22,9% w 2019 roku), jednak pod względem śmiertelności ustępuje rakowi oskrzela i płuca (rak oskrzela i płuca: 8 205 – 17,9%; rak piersi: 6 951 – 15,1%) [2]. W przeważającej części przypadków rak piersi diagnozowany jest w stadium naciekającym, którego dominującym typem histologicznym jest rak naciekający bez specjalnego typu (ang. *invasive carcinoma of no special type*; NST – 70-80% raków naciekających), dawniej nazywany rakiem przewodowym; drugi pod względem częstości występowania jest natomiast rak naciekający zrazikowy (ang. *invasive lobular carcinoma* – 10-15% raków naciekających) [3]. W ujęciu molekularnym raki piersi stanowią wysoce heterogenną grupę [4]. Na podstawie oceny ekspresji czterech markerów immunohistochemicznych: receptora estrogenowego (ER), receptora progesteronowego (PgR), receptora dla naskórkopochodnego czynnika wzrostu typu 2 (HER2) oraz indeksu proliferacyjnego Ki67 określa się podtyp molekularny: luminalny A, luminalny B HER2+, luminalny B HER2-, HER2+ lub potrójnie ujemny [5], który warunkuje rokowanie oraz dalszy dobór terapii.

Dzięki wprowadzeniu intensywnego programu przesiewowego raki piersi wykrywane są w coraz wcześniejszym stadium zaawansowania, jeszcze przed zajęciem węzłów chłonnych [6–9], najczęściej w niewielkim zaawansowaniu miejscowym– T1 [10]. Status zajęcia węzłów chłonnych (cecha N oceniana w ramach systemu TNM) określa się na podstawie biopsji węzła wartowniczego, tj. pierwszego węzła na drodze spływu chłonki z ogniska pierwotnego, który jest identyfikowany za pomocą limfoscyntygrafii. Obecność ogniska komórek nowotworowych w węzłach chłonnych wiąże się z podwyższonym ryzykiem nawrotu choroby – nawrót obserwowany jest w 70% przypadków zdiagnozowanych z zajętymi węzłami (stadium N+), w porównaniu do 30% nawrotu wśród chorych zdiagnozowanych z rakiem nierozsianym (stadium N-) [11,12]. U chorych na raka piersi wznowa występuje zwykle w postaci przerzutów odległych [13], które najczęściej spotykane są w kościach, płucu, wątrobie oraz mózgu [14].

Przerzuty odległe są najczęstszą przyczyną śmierci wśród chorych na złośliwe nowotwory [15,16], stąd rozsiew nowotworu postrzegany jest jako największe wyzwanie współczesnej onkologii. Formowanie przerzutów raka piersi może zachodzić dwutorowo – za pośrednictwem chłonki komórki raka mogą dostać się do węzłów chłonnych, podczas gdy przerzuty odległe w innych organach mogą tworzyć komórki wędrujące wraz z krwią. Powszechnie uważa się, że rozsiew droga limfatyczną i krwionośną to dwa rozdzielne i niezależne procesy [17], a obecność krążących komórek nowotworowych (ang. *circulating tumour cells*; CTC) w krwi obwodowej nie musi być związana z zajęciem węzłów chłonnych przez nowotwór [18,19]. Węzły chłonne na ogół są pierwszym organem zajętym przez komórki nowotworowe w trakcie rozsiewu. Wynika to z łatwości wejścia do układu chłonnego na skutek mniej ścisłej struktury naczyń limfatycznych w porównaniu do naczyń krwionośnych [20]. Co ważne, w ostatnim czasie pojawiają się doniesienia dowodzące możliwości formowania przerzutów odległych przez komórki ogniska przerzutowego w węźle chłonnym [21,22], co do tej pory było wyłącznie przedmiotem spekulacji. Najnowsze doniesienia na temat roli układu immunologicznego w przerzutowaniu wskazują, że po zajęciu węzłów chłonnych, pod wpływem interferonu oraz interakcji z komórkami układu odpornościowego, komórki nowotworowe przechodzą zmiany epigenetyczne, które zwiększają ich metastatyczny potencjał, a także wykształca się

swoista tolerancja układu immunologicznego wobec komórek nowotworowych, co dodatkowo sprzyja formowaniu przerzutów [23].

CTC uważane są za głównych inicjatorów przerzutów [24]. Kaskada metastatyczna jest wieloetapowym procesem, który obejmuje: oderwanie się komórek od masy guza pierwotnego, ich migrację przez przylegające tkanki aż do naczyń (krwionośnych lub limfatycznych), wejście do naczyń, drogę do docelowych organów (wraz z krwią lub chłonką), wyjście z naczyń i wreszcie zasiedlenie organu docelowego [25]. Podczas rozsiewu komórki nowotworowe często przechodzą tranzycję fenotypową – przejście epitelialno-mezenchymalne (ang. *epithelial-mesenchymal transition*; EMT). W trakcie tego procesu spolaryzowane, stacjonarne i epitelialne komórki przekształcają się w komórki mezenchymalne o zwiększonej mobilności i braku polarności [26]. EMT, fizjologiczny proces zachodzący w trakcie embriogenezy, jest zjawiskiem odwracalnym, a jego odwrotność nazywana jest tranzycją mezenchymalno-epitelialną (ang. *mesenchymal-epithelial transition*; MET) [27,28]. EMT ułatwia dotarcie CTC do docelowego organu, jednak zostało udowodnione, że do rozsiewu i uformowania ogniska przerzutowego konieczny jest powrót do fenotypu epitelialnego na drodze MET [29–31]. Zdolność do przechodzenia między fenotypami, określana mianem plastyczności fenotypowej, jest kluczowa w kontekście rozsiewu nowotworu [32–34]. Co ciekawe, część CTC utrzymuje tzw. status hybrydowy – epitelialno-mezenchymalny [19,35], co wiąże się z manifestacją cech charakterystycznych dla obu fenotypów [27]. Obecność CTC o fenotypie hybrydowym została powiązana ze szczególnie złą prognozą [36,37].

Indukcja EMT zachodzi pod wpływem szeregu czynników, które mogą być wydzielane m.in. przez komórki podścieliska guza, czyli tzw. mikrośrodowisko. Mikrośrodowisko guza składa się z wielu typów komórek, m.in. fibroblastów, komórek śródbłonka, perycytów, komórek układu odpornościowego – z których najliczniejsze są makrofagi, jak również substancji zewnątrzkomórkowej (ang. *extracellular matrix*; ECM), cytokin i czynników wzrostu [38]. Wszystkie te elementy wchodzą w ścisłą interakcję z komórkami nowotworowymi, a te oddziaływania z kolei odgrywają kluczową rolę w progresji i rozsiewie nowotworu [39]. Co więcej, zostało udowodnione, że mikrośrodowisko ewoluuje wraz z nowotworem, czyniąc ich interakcję niezwykle dynamiczną i złożoną [38]. Populacją dominującą wśród komórek mikrośrodowiska są fibroblasty, które nazywane są fibroblastami związanymi z nowotworem (ang. *cancer-associated fibroblasts*; CAFs). Prawidłowe fibroblasty są zaangażowane w liczne procesy, m.in. produkcję białek wchodzących w skład ECM, modulację odpowiedzi układu odpornościowego czy zachowanie homeostazy w tkance [40,41]. Z kolei działanie CAFs ma charakter pro-nowotworowy i pro-metastatyczny, co udowodniono także w raku piersi [42–44].

Podczas rozsiewu krążące komórki nowotworowe wchodzą w kontakt z elementami układu krążenia. Krew jest niewątpliwie niebezpiecznym i często niesprzyjającym z perspektywy CTC środowiskiem, z uwagi na oddziaływania fizyczne czy przeciwnowotworowe działanie układu odpornościowego [45]. Z drugiej jednak strony, zostało udowodnione, że płytki krwi, a także niektóre z obecnych w osoczu cytokin i czynników wzrostu, wspomagają CTC na ich drodze do organów odległych [38,46–48]. Płytki krwi wspomagają wyjście CTC z naczyń [49,50], modulują mechanizmy ucieczki spod kontroli układu odpornościowego [51], mogą również nasilać proliferację komórek nowotworowych [52]. Ponadto udowodnione zostało, że płytki zwiększają metastatyczny potencjał komórek nowotworowych [46,53], wpływając m.in. na proces EMT. Przedmiotem dyskusji pozostaje dokładna rola płytek krwi w EMT komórek nowotworowych – postuluje się, że są one odpowiedzialne za indukcję całego procesu lub też za jego podtrzymanie [53,54].

Większość oddziaływań guz–mikrośrodowisko odbywa się z udziałem elementów układu immunologicznego. Układ odpornościowy, którego głównym zadaniem jest ochrona organizmu przed patogenami, odgrywa istotną rolę w rozwoju i progresji nowotworu. Komórki nowotworowe pozostają w stałej interakcji z komórkami układu immunologicznego, która może prowadzić zarówno do stymulacji, jak i zahamowania wzrostu guza [55]. Jedną z cech charakterystycznych komórek nowotworowych jest umiejętność ucieczki spod kontroli układu odpornościowego [56], która, jak udowodniono, może być dodatkowo wspomagana przez komórki mikrośrodowiska [57,58].

Podejmowane dotąd próby farmakologicznej deplecji komórek podścieliska guza przyniosły niezadowalający efekt [59]. Zasadna wydaje się natomiast ich modulacja, tzw. re-edukacja, mające na celu nasilenie przeciwnowotworowego działania podścieliska. Do takiego celu może posłużyć immunoterapia, która od momentu wprowadzenia w 2011 znacznie poprawiła skuteczność leczenia wielu nowotworów [60–62]. Dogłębne zrozumienie interakcji guz-mikrośrodowisko i ich znaczenia dla rozsiewu raka piersi może doprowadzić do identyfikacji nowych celów dla immunoterapii, a także dostarczyć wskazań do jej zastosowania.

Poznanie molekularnych mechanizmów procesu tworzenia przerzutów jest niezwykle istotne dla rozwoju skutecznych metod detekcji rozsiewu, oceny ryzyka nawrotu choroby czy wreszcie jej leczenia. Dostępne dane literaturowe nie pozwalają w sposób jednoznaczny zdefiniować udziału układu immunologicznego we wczesnym rozsiewie raka piersi, wskazując na jego dwoistą rolę; z jednej strony komórki układu immunologicznego są zdolne do rozpoznawania i eliminacji komórek nowotworowych, z drugiej natomiast, przewlekły stan zapalny sprzyja rozwojowi guza [55].

Cele pracy

Zasadniczym celem pracy było opisanie związku pomiędzy profilem transkrypcyjnym raka piersi, obejmującym geny związane z układem immunologicznym – tzw. immuno-transkryptomem a przebiegiem choroby, ze szczególnym uwzględnieniem zajęcia węzłów chłonnych, obecności krążących komórek nowotworowych (CTC), liczby płytek krwi oraz fenotypem epitelialno-mezenchymalnym komórek nowotworowych na różnych etapach rozsiewu na drodze limfatycznej i krwionośnej.

Przedmiotem analizy były pierwotne guzy (ang. *primary tumours*, PT) raka piersi o zbadanym statusie zajęcia węzłów chłonnych (niezajęte, N-; zajęte, N+) oraz sparowane przerzuty do węzłów chłonnych (ang. *lymph node metastases*; LNM).

Cele szczegółowe:

I. Określenie profilu transkrypcyjnego związanego z układem immunologicznym guzów pierwotnych w odniesieniu do fenotypu krążących komórek nowotworowych (mezenchymalny vs. epitelialny) w krwi obwodowej **[P1]**.

II. Określenie profilu transkrypcyjnego związanego z układem immunologicznym guzów pierwotnych w odniesieniu do liczby płytek krwi w krwi obwodowej **[P2]**.

III. Określenie profilu transkrypcyjnego związanego z układem immunologicznym:

    a. guzów pierwotnych, które nie wytworzyły przerzuty do węzłów chłonnych;
    b. guzów pierwotnych, które wytworzyły przerzuty do węzłów chłonnych;
    c. przerzutów do węzłów chłonnych;

    w odniesieniu do fenotypu występujących w nich komórek nowotworowych (mezenchymalny vs. epitelialny) **[P3]**.

IV. Określenie profilu transkrypcyjnego związanego z układem immunologicznym guzów pierwotnych w odniesieniu do sparowanych przerzutów do węzłów chłonnych – rozsiew drogą limfatyczną **[P4]**.

Omówienie publikacji wchodzących w skład rozprawy doktorskiej

Wymienione w poprzednim rozdziale cele szczegółowe rozprawy zostały ujęte w czterech publikacjach oryginalnych [P1-4] opublikowanych w międzynarodowych czasopismach indeksowanych na Liście Filadelfijskiej.

Cel I: Określenie profilu transkrypcyjnego związanego z układem immunologicznym guzów pierwotnych w odniesieniu do fenotypu krążących komórek nowotworowych (mezenchymalny vs. epitelialny) w krwi obwodowej.

Publikacja 1 [P1]

Popeda M, Stokowy T, Bednarz-Knoll N, Jurek A, Niemira M, Bielska A, Kretowski A, Kalinowski L, Szade J, Markiewicz A, Zaczek AJ. **NF-kappa B Signaling-Related Signatures Are Connected with the Mesenchymal Phenotype of Circulating Tumor Cells in Non-Metastatic Breast Cancer**. Cancers (Basel). 2019 Dec 6;11(12):1961. doi: 10.3390/cancers11121961

Dostępne dane literaturowe wskazują na udział układu immunologicznego w procesie przerzutowania, jednak brak jest precyzyjnych doniesień na temat około-immunologicznych mechanizmów warunkujących formowanie oraz fenotyp epitelialno-mezenchymalny krążących komórek nowotworowych. W pracy zbadaliśmy związek pomiędzy profilem transkrypcyjnym guza pierwotnego w obrębie genów związanych z układem immunologicznym a obecnością CTC w krwioobiegu i ich fenotypem.

W tym celu przeprowadziłam profilowanie ekspresji 730 genów związanych z układem immunologicznym (nCounter PanCancer Immune Profiling Panel) za pomocą technologii nCounter (NanoString). Grupę badaną stanowiło 35 pierwotnych guzów piersi w stadium I-III (nierozsianym) o znanym przedoperacyjnym statusie CTC [63]. W skrócie, fenotyp CTC został określony na podstawie ekspresji RNA dwóch markerów – *CK19* (*Cytokeratin 19*; inaczej *KRT19*, *Keratin 19*) oraz *VIM* (*Vimentin*); CTC o fenotypie CK19(+)/VIM(-) były klasyfikowane jako epitelialne, natomiast CK19(-)/VIM(+) jako mezenchymalne. Ekstrakcję RNA przeprowadziłam na dostępnym archiwalnym materiale tkankowym utrwalonym w formalinie i zatopionym w parafinie (ang. *formalin-fixed, paraffin-embedded*; FFPE). Technologia nCounter jest uważana za złoty standard analiz transkryptomicznych w materiale typu FFPE, który charakteryzuje się wysokim stopniem degradacji kwasów nukleinowych. Technologia jest oparta na zastosowaniu multipleksowej mieszaniny krótkich, wyznakowanych fluorescencyjnie sond, ich hybrydyzacji do cząsteczek RNA w izolacie, a następnie detekcji i zliczeniu sygnału fluorescencyjnego. Analiza metodą nCounter została wykonana w ramach współpracy z Centrum Badań Klinicznych Uniwersytetu Medycznego w Białymstoku.

Pozyskane dane surowe w postaci liczby odczytów sond w próbce poddałam obróbce bioinformatycznej w dedykowanym oprogramowaniu – nSolver 4.0 (NanoString). W pierwszym kroku dane zostały poddane kontroli jakości, która wykluczyła próbki o niskich parametrach z dalszych analiz. Następnie wykonałam korekcję tła w oparciu o wartości odczytów dla ujemnych sond kontrolnych, które charakteryzują się brakiem komplementarności

do sekwencji występujących w ludzkim DNA. Korekcję wykonałam w sposób rekomendowany dla danych przeznaczonych do analiz krotności zmiany ekspresji genu (ang. *fold change*, FC), tj. poprzez progowanie tła. W kolejnym kroku przeprowadziłam normalizację danych w oparciu o wartości odczytów dla dodatnich sond kontrolnych, które pozwalają ocenić skuteczność hybrydyzacji, oraz wartości odczytów dla genów referencyjnych o największej stabilności ekspresji, które wyłonił zastosowany algorytm NormFinder [64]. Znormalizowane wartości ekspresji genów zostały zdeponowane w repozytorium NCBI GEO [65] pod numerem dostępu GSE180186 wraz z podstawową charakterystyką kliniczną chorych.

Właściwą różnicową analizę porównawczą genów przeprowadziłam z wykluczeniem genów o niskiej ekspresji (log2 średniej odczytów we wszystkich próbkach poniżej 6). Zależną od danej cechy różnicę ekspresji genu wyraziłam w postaci krotności zmiany obliczonej dla median liczby odczytów w porównywanych grupach, a następnie określiłam jej istotność statystyczną za pomocą testu Manna-Whitneya-Wilcoxona. Z uwagi na znaczną liczbę (584) jednocześnie analizowanych genów, zastosowałam poprawkę na testowanie wielokrotne metodą Benjaminiego-Hochberga.

W przebiegu przeprowadzonej analizy nie zidentyfikowałam genów o statystycznie znamiennym powiązaniu z obecnością CTC w krwioobiegu. Natomiast analogiczne porównanie przeprowadzone dla PT chorych, u których wykryto CTC o fenotypie mezenchymalnym oraz epitelialnym, wskazało na statycznie znamienne, zależne od fenotypu CTC różnice w ekspresji 38 genów (ang. *differentially expressed genes*; DEGs). Guzy rozsiewające mezenchymalne CTC wykazały wyższą ekspresję 37 genów, które były związane ze ścieżką sygnalizacyjną NF-kB, a także powiązane z produkcją interferonów typu I, co wykazała analiza adnotacji funkcjonalnych wykonana w bazie DAVID Bioinformatics Resources 6.8 [66,67] za pomocą narzędzia *Functional Annotation Tool* oraz przegląd danych literaturowych. Natomiast guzy rozsiewające epitelialne CTC charakteryzowała podwyższona ekspresja genu *MERTK* (*MER Proto-Oncogene, Tyrosine Kinase*), który jest zaangażowany m.in. w hamowanie mechanizmów odporności wrodzonej czy sygnalizacji zależnej od cytokin. Należy podkreślić, że zidentyfikowane geny związane ze szlakiem sygnalizacyjnym NF-kB plasują się na wielu jego poziomach – od ligandów, przez ich receptory, białka adaptorowe, przekaźniki sygnału, czynniki transkrypcyjne, aż po regulowane geny, co przedstawia **Rycina 3** w **[P1]**. Świadczy to o istotnej modulacji całej ścieżki sygnałowej NF-kB w zależności od statusu EMT generowanych CTC.

Jako, że nasze wcześniejsze badania wykazały negatywny wpływ prognostyczny CTC o fenotypie mezenchymalnym, w kolejnym kroku postanowiłam zbadać wpływ poziomu ekspresji zidentyfikowanych genów, które ulegały różnicowej ekspresji pomiędzy fenotypami, na przeżycie. Do tego celu wykorzystałam publicznie dostępne dane kliniczne i transkryptomiczne zgromadzone dla grupy chorych z inwazyjnym rakiem piersi w ramach projektu The Cancer Genome Atlas (TCGA) [68]. W analizie metodą modelu proporcjonalnego hazardu Coxa, 5 spośród 37 zidentyfikowanych genów o podwyższonej ekspresji w PT rozsiewających CTC o fenotypie mezenchymalnym wykazało negatywny wpływ na przeżycie: jeden na czas przeżycia całkowitego (OS, n=876) – *PSMD7* (*Proteasome 26S Subunit, Non-ATPase 7*; HR=1,75, p=0,022; HR, ang. *hazard ratio*), natomiast cztery na czas przeżycia wolnego od choroby (DFS, n=701): *C2* (*Complement C2*; HR=4,51, p=0,014), *IFNAR1* (*Interferon Alpha And Beta Receptor Subunit 1*; HR=2,68, p=0,043), *CD84* (*CD84 Molecule*; HR=2,48, p=0,012) i *CYLD* (*CYLD Lysine 63 Deubiquitinase*; HR=2,20, p=0,028); związek z przeżyciem potwierdziła również analiza wieloczynnikowa oparta na modelu regresji uwzględniającym stadium zaawansowania choroby.

Podsumowując, w pracy wykazaliśmy, że immuno-transkryptom guza pierwotnego nie jest związany z obecnością, ale ze statusem epitelialno-mezenchymalnym CTC obecnych w krwioobiegu. Podwyższona ekspresja genów związanych z sygnalizacją czynnika transkrypcyjnego NF-kB towarzyszy mezenchymalnemu fenotypowi komórek krążących, a niektóre z nich stanowią negatywny czynnik prognostyczny. Nasze wyniki wskazują szlak sygnalizacyjny NF-kB jako interesujący cel dla nowych rozwiązań terapeutycznych, mających na celu zapobieganie rozsiewowi raka piersi i nawrotom choroby.

Cel II: Określenie profilu transkrypcyjnego związanego z układem immunologicznym guzów pierwotnych w odniesieniu do liczby płytek krwi w krwi obwodowej.

Publikacja 2 [P2]

Bednarz-Knoll N, **Popęda M**, Kryczka T, Kozakiewicz B, Pogoda K, Szade J, Markiewicz A, Strzemecki D, Kalinowski L, Skokowski J, Liu J, Żaczek AJ. **Higher platelet counts correlate to tumour progression and can be induced by intratumoural stroma in non-metastatic breast carcinomas**. Br J Cancer. 2022 Feb;126(3):464-471. doi: 10.1038/s41416-021-01647-9

Rola płytek krwi w rozsiewie nowotworów jest bezsprzeczna, jednak nadal brak jest konkretnych doniesień na temat ich interakcji z guzem pierwotnym, jego mikrośrodowiskiem, krążącymi komórkami nowotworowymi oraz układem immunologicznym u chorych na raka piersi. W pracy podjęliśmy próbę kompleksowej charakterystyki tych oddziaływań wraz z określeniem wpływu liczby płytek krwi na prognozę. Grupę badaną stanowiły chore na operacyjnego raka piersi w stadium I-III (n=70), dla których dostępne były przedoperacyjne wyniki badań krwi, w tym liczba płytek krwi, przedoperacyjny status krążących komórek nowotworowych (jak opisano w Markiewicz i wsp. [63]), dane odnośnie przeżycia całkowitego, a także ocena obfitości podścieliska w guzie pierwotnym. Dodatkowo dla części grupy dysponowaliśmy danymi nt. immuno-transkryptomu guza pierwotnego (n=32, jak opisano w **[P1]**) oraz wynikami multipleksowego profilowania poziomu 30 cytokin w osoczu (n=36) za pomocą technologii Luminex xMAP (Bio-Rad) oraz dedykowanego zestawu 65-Plex Human ProcartaPlex TM Panel (Invitrogen).

Dowiedliśmy, że górny kwartyl zakresu normy liczby płytek krwi w krwioobiegu (ang. *high normal platelet count*; hPC – powyżej 289 000/µl; zakres normy 150-400 000/µl [69]) był związany z szeregiem cech kliniczno-patologicznych, tj. wyższym statusem T, wyższym stopniem złośliwości (ang. *grade*) oraz mezenchymalnym fenotypem CTC. Co ciekawe, chore, u których wykryto CTC (bez względu na ich fenotyp) wykazały wyższą liczbę płytek krwi w porównaniu do chorych CTC-ujemnych, jednak różnica ta nie osiągnęła istotności statystycznej (p=0,125).

W grupie chorych z zajętymi węzłami chłonnymi status hPC korelował również z większą liczbą zajętych węzłów. W kontekście prognostycznym, status hPC był związany z krótszym czasem całkowitego przeżycia zarówno samodzielnie (HR=9,635, p=0,001), jak i w połączeniu z obecnością CTC w krwioobiegu (HR=9,452, p=0,001) bez względu na ich status EMT. Negatywny wpływ hPC na prognozę potwierdziliśmy również w niezależnej grupie walidacyjnej, obejmującej chore na raka o podtypie luminalnym (n=254; HR=3,626, p=0,021). Aby lepiej zrozumieć sieć możliwych zależności pomiędzy płytkami krwi, CTC a guzem pierwotnym, przeanalizowaliśmy poziom cytokin osoczowych, wykazując statystycznie znamienny związek pomiędzy statusem hPC a podwyższonym stężeniem 8 cytokin – APRIL (TNFSF13), ENA78 (CXCL5), HGF, IL16, IL17A, MCP3, MDC (CCL22) oraz SCF. Co istotne, niektóre z tych cytokin (APRIL [TNFSF13] [70], HGF [71], IL17A [72] i SCF [73]) mogą być powiązane z trombopoezą. Ponadto, guzy chorych ze statusem hPC wykazały większą obfitość podścieliska.

Chcąc zgłębić charakterystykę podścieliska, wykonałam analizę składu populacyjnego nacieku inflitrującego guz, bazując na wykonanym uprzednio profilowaniu immuno-tranksryptomu guzów pierwotnych **[P1]**. W tym celu wykorzystałam metodę cytometrii cyfrowej

(ang. *digital cytometry*) – algorytm CIBERSORTx [74]. Pozwala on m.in. oszacować absolutny skład populacyjny mieszaniny komórek (analiza zbiorcza, ang. *bulk*) na podstawie profilu transkryptomicznego, bazując na porównaniu z wzorcową macierzą ekspresji genów w 22 typach komórek hematopoetycznych.

Wygenerowane dane w postaci proporcjonalnej wartości udziału danej frakcji komórek w próbce poddałam analizie porównawczej, popartej analizą statystyczną z zastosowaniem testu Manna-Whitneya-Wilcoxona. W ten sposób wykazałam, że guzy chorych o statusie hPC, w porównaniu do chorych o statusie nPC (ang. *normal platelet count* – zakres normy liczby płytek w krwioobiegu poniżej górnego kwartyla, 289 000/µl), wykazują znamiennie wyższy udział dwóch populacji komórek – limfocytów T CD8+ (FC=1,88, p=0,026) oraz komórek tucznych w fazie spoczynku (FC=1,54, p=0,041). Co istotne, guzy z większą infiltracją tych 2 typów komórek prezentowały również wyższą ekspresję RNA kodujących cytokiny, których wysokie poziomy zaobserwowaliśmy w osoczu chorych o statusie hPC – IL17A (*IL17A*; p=0,033) w przypadku komórek tucznych oraz MMP1 (*MMP1*; p=0,01) i MDC (*CCL22*; p=0,01) w przypadku limfocytów T CD8+.

Podsumowując, w pracy wykazaliśmy, że profil immuno-transkryptomiczny powiązany z górnym kwartylem zakresu normy liczby płytek krwi w krwioobiegu wskazuje na zwiększoną infiltrację guza przez CD8-dodatnie limfocyty T oraz komórki tuczne w fazie spoczynku. Dodatkowo, guzy te wykazały podwyższoną ekspresję RNA kodujących cytokiny – IL17A (*IL17A*), MDC (*CCL22*) oraz MMP1 (*MMP1*). Postulujemy, że cytokiny te mogą wzmagać produkcję płytek w szpiku kostnym, nasilać ich aktywację oraz pro-nowotworową aktywność, a co za tym idzie wspierać rozsiew nowotworu drogą krwionośną.

Cel III: Określenie profilu transkrypcyjnego związanego z układem immunologicznym: guzów pierwotnych, które nie wytworzyły przerzutów do węzłów chłonnych; guzów pierwotnych, które wytworzyły przerzuty do węzłów chłonnych; przerzutów do węzłów chłonnych; w odniesieniu do fenotypu występujących w nich komórek nowotworowych (mezenchymalny vs. epitelialny).

Publikacja 3 [P3]

Markiewicz A, Topa J, **Popęda M**, Szade J, Skokowski J, Wełnicka-Jaśkiewicz M, Żaczek A. **Activation of epithelial-mesenchymal transition process during breast cancer progression – the impact of molecular subtype and stromal composition**. Acta Biochim Pol. 2021 Aug 25;68(3):385-392. doi: 10.18388/abp.2020_5719

Przejście epitelialno-mezenchymalne przyczynia się do zwiększenia agresywności komórek nowotworowych, a co za tym idzie, ułatwia rozsiew guza. Udowodniono, że układ immunologiczny może indukować EMT, jednak związek pomiędzy statusem EMT a immuno-transkryptomem pierwotnych guzów piersi oraz ich ognisk przerzutowych w węzłach chłonnych pozostaje niedostatecznie poznany. W pracy skupiliśmy się na operacyjnych rakach w stadium I-III (n=88), o podtypie luminalnym oraz potrójnie ujemnym oraz ich statusie EMT, definiowanym na podstawie oceny ekspresji 3 białek – E-kadheryny (E-cad), N-kadheryny (N-cad) oraz wimentyny (Vim), jak opisano w Markiewicz i wsp. [75]; fenotyp E-cad(+)/N-cad(-)/Vim(-) był klasyfikowany jako epitelialny, natomiast fenotyp mezenchymalny stwierdzano w przypadku tkanek E-cad(-) lub N-cad(+) lub Vim(+).

Badanie miało na celu identyfikację różnic w aktywacji EMT w guzach pierwotnych, w zależności od statusu węzłów chłonnych, który oddaje zdolność guza pierwotnego do rozsiewu (PT N- vs. PT N+), jak również w przerzutach do węzłów chłonnych (LNM), z uwzględnieniem różnorodności związanej z podtypem molekularnym. Guzy luminalne, które nie wytworzyły przerzutów do węzłów chłonnych (PT N-), wykazały najniższy odsetek aktywacji EMT (27%) oraz znaczną różnicę względem guzów, które przerzutowały do węzłów chłonnych (PT N+, 48%; p=0,06). Z kolei wśród raków potrójnie ujemnych aktywacja EMT była częsta, ale wykazała nieznaczne i nieznamienne zróżnicowanie względem statusu zajęcia węzłów chłonnych (83% PT N-, 63% PT N+). Co ciekawe, przerzuty do węzłów chłonnych wykazały podobny odsetek aktywacji EMT (32%) oraz zróżnicowanie w zależności od podtypu molekularnego (24% podtyp luminalny, 63% podtyp potrójnie ujemny; p=0,08), jak guzy pierwotne, które nie wytworzyły przerzutów do węzłów chłonnych (PT N-).

Z uwagi na potencjalny udział mikrośrodowiska i czynników związanych z układem immunologicznym w indukcji EMT, przeprowadziłam analizę immuno-transkryptomu w zależności od statusu EMT i statusu zajęcia węzłów chłonnych dla guzów luminalnych, które wykazały interesującą różnicę w zakresie aktywacji EMT. W analizie wykorzystałam dane dla guzów pierwotnych **[P1]** oraz węzłów chłonnych. Dane dla LNM wygenerowałam analogicznie jak w przypadku guzów pierwotnych (szczegółowy opis metodologii jak dla **[P1]**); w skrócie profilowanie ekspresji genów związanych z układem immunologicznym wykonałam za pomocą technologii nCounter (NanoString) dla 11 przerzutów do węzłów chłonnych. Obróbkę bioinformatyczną oraz normalizację danych wykonałam w oprogramowaniu nSolver 4.0

(NanoString). Dane transkryptomiczne LNM również zostały udostępnione w repozytorium NCBI GEO pod wspólnym numerem dostępu GSE180186.

W różnicowej analizie ekspresji genów z dalszą analizą adnotacji funkcjonalnych (jak opisano dla **[P1]**) wykazałam, że guzy N- o fenotypie mezenchymalnym charakteryzują się obniżoną ekspresję genów związanych z odpowiedzią zapalną, z istotnie obniżoną ekspresją (log2FC≤-1 oraz p<0.05) pięciu genów: *PRG2* (*Proteoglycan 2, Pro Eosinophil Major Basic Protein*), *PTGS2* (*Prostaglandin-Endoperoxide Synthase 2*), *IFNA7* (*Interferon Alpha 7*), *ITGA2B* (*Integrin Subunit Alpha 2b*) oraz *CXCL3* (*C-X-C Motif Chemokine Ligand 3*). Jedynie gen *CD164* (*CD164 Molecule*) wykazał podwyższenie ekspresji (log2FC=1,05, p=0,042) w guzach mezenchymalnych. Z kolei guzy przerzutujące do węzłów chłonnych (PT N+) wykazały zależne od fenotypu EMT zróżnicowanie w zakresie genów zaangażowanych w procesowanie i prezentację antygenów oraz sygnalizację związaną z limfocytami T; dwa geny wykazały wyjątkowo wysoki wzrost ekspresji – *HLA-DQA1* (*Major Histocompatibility Complex, Class II, DQ Alpha 1*; log2FC=8,38, p=0,013) oraz *HLA-DQB1* (*Major Histocompatibility Complex, Class II, DQ Beta 1*; log2FC=6,49, p=0,016). Należy podkreślić, że guzy N+ wykazały znaczącą różnicę w obfitości komponentu stromalnego, która była większa w przypadku guzów o fenotypie mezenchymalnym. W połączeniu z wynikiem analizy transkryptomicznej może to wskazywać na wyższą infiltrację guzów N+ przez komórki prezentujące antygen. W przypadku LNM zaobserwowałam zróżnicowanie ekspresji genów powiązanych z produkcją interferonu, z największym spadkiem ekspresji w przerzutach mezenchymalnych odnotowanym dla genu *IFITM2* (*Interferon Induced Transmembrane Protein 2*; log2FC=-1,11, p=0,006).

Podsumowując, w pracy wykazaliśmy, że profil transkrypcyjny genów związanych z układem immunologicznym w rakach piersi o podtypie luminalnym jest zależny od statusu EMT, zdolności guza pierwotnego do rozsiewu oraz etapu kaskady metastatycznej. W guzach nierozsianych (PT N-) fenotyp mezenchymalny był związany z obniżeniem ekspresji genów związanych z reakcją zapalną. Mezenchymalne guzy zdolne do tworzenia przerzutów do węzłów chłonnych (PT N+) wykazały natomiast podwyższoną aktywność, a potencjalnie również zwiększoną infiltrację, komórek prezentujących antygen, natomiast po zajęciu węzłów chłonnych (LNM) komórki nowotworowe o statusie mezenchymalnym wykazały obniżenie ekspresji genów związanych z produkcją interferonu. Co istotne, geny o różnicowej ekspresji zidentyfikowane w ramach tych trzech porównań (mezenchymalny vs. epitelialny dla PT N-, PT N+ oraz LNM) nie pokrywały się ze sobą, wskazując na zupełnie odmienny, zależny od stopnia rozsiewu nowotworu, sposób odziaływania mikrośrodowiska guza na komórki nowotworowe i ich tranzycję fenotypową.

Cel IV: Określenie profilu transkrypcyjnego związanego z układem immunologicznym guzów pierwotnych w odniesieniu do sparowanych przerzutów do węzłów chłonnych – rozsiew drogą limfatyczną.

Publikacja 4 [P4]

Popeda M, Markiewicz A, Stokowy T, Szade J, Niemira M, Kretowski A, Bednarz-Knoll N, Zaczek AJ. **Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients**. Sci Rep. 2021 Mar 3;11(1):5097. doi: 10.1038/s41598-021-84568-0

Układ immunologiczny niewątpliwie odgrywa istotną rolę w formowaniu przerzutów raka piersi, jednak powiązany z nim profil transkrypcyjny jak dotąd nie został opisany w kontekście rozsiewu. Celem pracy była identyfikacja zmian w obrębie immuno-transkryptomu w przerzutach do węzłów chłonnych względem sparowanych guzów pierwotnych. Analiza objęła 11 par: przerzut do węzłów chłonnych (LNM) oraz guz pierwotny o podtypie luminalnym (PT N+), których profile transkrypcyjne zostały ustalone, jak opisano odpowiednio dla **[P1]** i **[P3]**.

Z uwagi na fizjologiczne różnice pomiędzy tkanką piersi i węzłów chłonnych, w analizie danych mającej na celu porównanie LNM do PT uwzględniłam etap korekcji względem tkanek prawidłowych (schemat analizy szczegółowo ilustruje **Rycina 1** w **[P4]**). W tym celu z bazy GeneCards [76] pozyskałam profil transkryptomiczny prawidłowej piersi oraz prawidłowego węzła chłonnego, które zostały wygenerowane w ramach projektu Genotype-Tissue Expression (GTEx) [77]. Z uwagi na niekompatybilność zastosowanych metod analizy – nCounter w immuno-traksryptomicznych danych własnych, sekwencjonowanie RNA (ang. *RNA-seq*) w danych z projektu GTex, konieczne było wykonanie korekcji sparowanych współczynników (ang. *ratio*) tkanek nowotworowych (LNM vs. PT N+) względem współczynników tkanek prawidłowych (węzeł chłonny vs. pierś). Dla 360 genów dostępnych w obu zbiorach obliczyłam medianę skorygowanych sparowanych współczynników LNM vs. PT N+, która następnie posłużyła do selekcji genów o podwyższonej (11 genów, w tym markery tkanki piersiowej, raka piersi lub geny o udowodnionym związku z przerzutowaniem) i obniżonej (70 genów, w tym głównie elementy odporności wrodzonej) ekspresji w LNM względem prawidłowego węzła chłonnego. W kolejnym kroku wyodrębniłam geny o podwyższonej/obniżonej ekspresji w LNM względem PT N+, w liczbie odpowiednio 3 i 33. Trzy geny o podwyższonej ekspresji w LNM – *ATG10* (*Autophagy Related 10*), *GATA3* (*GATA Binding Protein 3*) oraz *S100B* (*S100 Calcium Binding Protein B*), można uznać za potencjalne markery zwiększonej agresywności i potencjału komórek raka piersi do przerzutowania. Natomiast geny o obniżonej ekspresji w LNM były związane głównie z układem dopełniacza, co wykazała analiza adnotacji funkcjonalnych wg bazy ConsensusPathDB [78]. Ścisła siatka powiązań pomiędzy zidentyfikowanymi genami znalazła potwierdzenie w wizualizacji interakcji molekularnych, którą wykonałam w bazie STRING [79].

W celu weryfikacji tej obserwacji na poziomie białka wykonałam immunohistochemiczne oznaczenie białka C3 – centralnego elementu układu dopełniacza, w mikromacierzach tkankowych obejmujących materiał 79 chorych z luminalnym rakiem piersi (PT N-, n=36; PT N+, n=43; LNM, n=43). Ekspresja białka C3 została zaklasyfikowana jako pozytywna lub negatywna osobno dla komponentu nowotworowego i stromalnego na podstawie oceny odczynu w co najmniej 3 z 5 fragmentów tkanki. Oznaczenie było

informatywne dla 35 par PT N+ i LNM. Co ważne, w grupie chorych z zajętymi węzłami chłonnymi zaobserwowałam statystycznie istotne obniżenie odsetka LNM wykazujących ekspresję białka C3 w komórkach nowotworowych w porównaniu do PT N+ (p=0,006), natomiast takie różnice nie wystąpiły w przypadku ekspresji C3 w podścielisku (p=1). Dodatkowo, brak ekspresji C3 w komórkach nowotworowych w LNM był związany z gorszą prognozą w zakresie 3-letniego przeżycia całkowitego (p=0,01).

Podsumowując, w pracy wykazaliśmy, że ekspresja genów związanych z układem dopełniacza ulega obniżeniu w komórkach LNM względem komórek PT pochodzących od tych samych chorych. Analogiczna zmiana zachodzi również w obrębie białka C3 – centralnego elementu układu dopełniacza, które ulega lokalnej ekspresji w komórkach nowotworowych guza pierwotnego, lecz zanika w komórkach w obrębie ognisk przerzutowych w węzłach chłonnych. Układ dopełniacza wydaje się być istotnym czynnikiem zdolnym do lokalnej modulacji rozsiewu raka piersi, a przez to jawi się jako potencjalny cel terapii przeciwdziałających powstawaniu przerzutów.

Podsumowanie i dyskusja

Przerzuty są najczęstszą przyczyną śmierci wśród chorych na nowotwory. Rozsiew nowotworu to złożony proces, w który zaangażowane są zarówno komórki guza, jak również komórki otaczającego go mikrośrodowiska, w tym komórki układu immunologicznego. Istotne są również bezpośrednie i pośrednie oddziaływania, w które komórki guza wchodzą z elementami układu krążenia, np. płytkami krwi czy osoczem i przekazywanymi za jego pośrednictwem cytokinami. Niniejsza rozprawa skupia się na najczęstszym nowotworze wśród kobiet – raku piersi oraz około-immunologicznych mechanizmach towarzyszących jego rozsiewowi (**Rycina 1**).

Rozwój metod analiz transkryptomicznych dedykowanych dla materiału utrwalonego w formalinie i zatopionego w parafinie był przełomowy dla postępu onkologii translacyjnej. Dzięki wprowadzeniu technologii takich jak nCounter (NanoString), a także gwałtownemu postępowi w dziedzinie bioinformatyki i pojawianiu się nowych narzędzi zwiększających zasób informacji, które można wyekstrahować z danych transkryptomicznych, obecnie możliwe jest uzyskanie znacznej informacji biologicznej z materiału archiwalnego. Badania ujęte w mojej rozprawie doktorskiej zostały przeprowadzone na materiale klinicznym, który został zgromadzony w latach 2010-2012 i od tamtej pory był poddawany sukcesywnej, wielopłaszczyznowej analizie w zespole Promotor pracy – prof. dr hab. Anny J. Żaczek. Przeprowadzona przeze mnie analiza immuno-transkryptomu pozwoliła pogłębić zrozumienie wcześniejszych obserwacji. Zaproponowane wnioski mają charakter nowatorski i stanowiły pierwsze tego typu doniesienia na temat profili transkryptomicznych związanych z układem odpornościowym w kontekście rozsiewu operacyjnego raka piersi.

Pierwszym odkryciem było wykazanie związku pomiędzy podwyższoną ekspresją genów związanych z sygnalizacją czynnika transkrypcyjnego NF-kB w guzie pierwotnym a mezenchymalnym fenotypem rozsiewanych przez niego komórek krążących **[P1]**. Szlak sygnalizacyjny NF-kB jest znanym induktorem przejścia epitelialno-mezenchymalnego, zarówno w warunkach fizjologicznych, jak i w nowotworzeniu [80]. Udowodniony został również jego wpływ na zwiększoną proliferację, oporność na leczenie, czy wreszcie zdolność formowania przerzutów przez komórki raka piersi [81]. Co istotne, NF-kB bezpośrednio reguluje transkrypcję genów kodujących czynniki transkrypcyjne, które stymulują EMT, tj. *TWIST1* (*Twist Family BHLH Transcription Factor 1*), *SLUG* (*SNAI2*; *Snail Family Transcriptional Repressor 2*) i *SIP1* (*ZEB2; Zinc Finger E-Box Binding Homeobox 2*) [82]. W naszej pracy po raz pierwszy wykazaliśmy związek pomiędzy nasiloną ekspresją genów związanych z sygnalizacją NF-kB, co istotne – plasujących się na wielu poziomach ścieżki, a statusem EMT krążących komórek nowotworowych. Ta obserwacja stanowi ciekawe uzupełnienie badań Labelle i współpracowników, którzy wykazali w modelach *in vitro* i *in vivo*, że pod wpływem interakcji z płytkami w komórkach nowotworowych aktywacji ulega szlak NF-kB, co prowadzi do indukcji EMT, a także zwiększa skuteczność formowania przerzutów[54]. Warte podkreślenia jest również, że wśród zidentyfikowanych przez nas genów o ekspresji związanej z fenotypem rozsiewanych CTC znalazły się markery aktywacji płytek krwi – *CD63* (*CD63 Molecule*) i *CD84* (*CD84 Molecule*).

W drugiej pracy **[P2]**, poświęconej badaniu płytek krwi, ich związku z CTC i rokowaniem, postawiliśmy tezę, że podwyższona liczba płytek krwi w krwioobiegu może wynikać z zachodzących w obrębie guza procesów, w które zaangażowane są elementy układu immunologicznego. Mianowicie, guz pierwotny, za pośrednictwem swojego obfitego

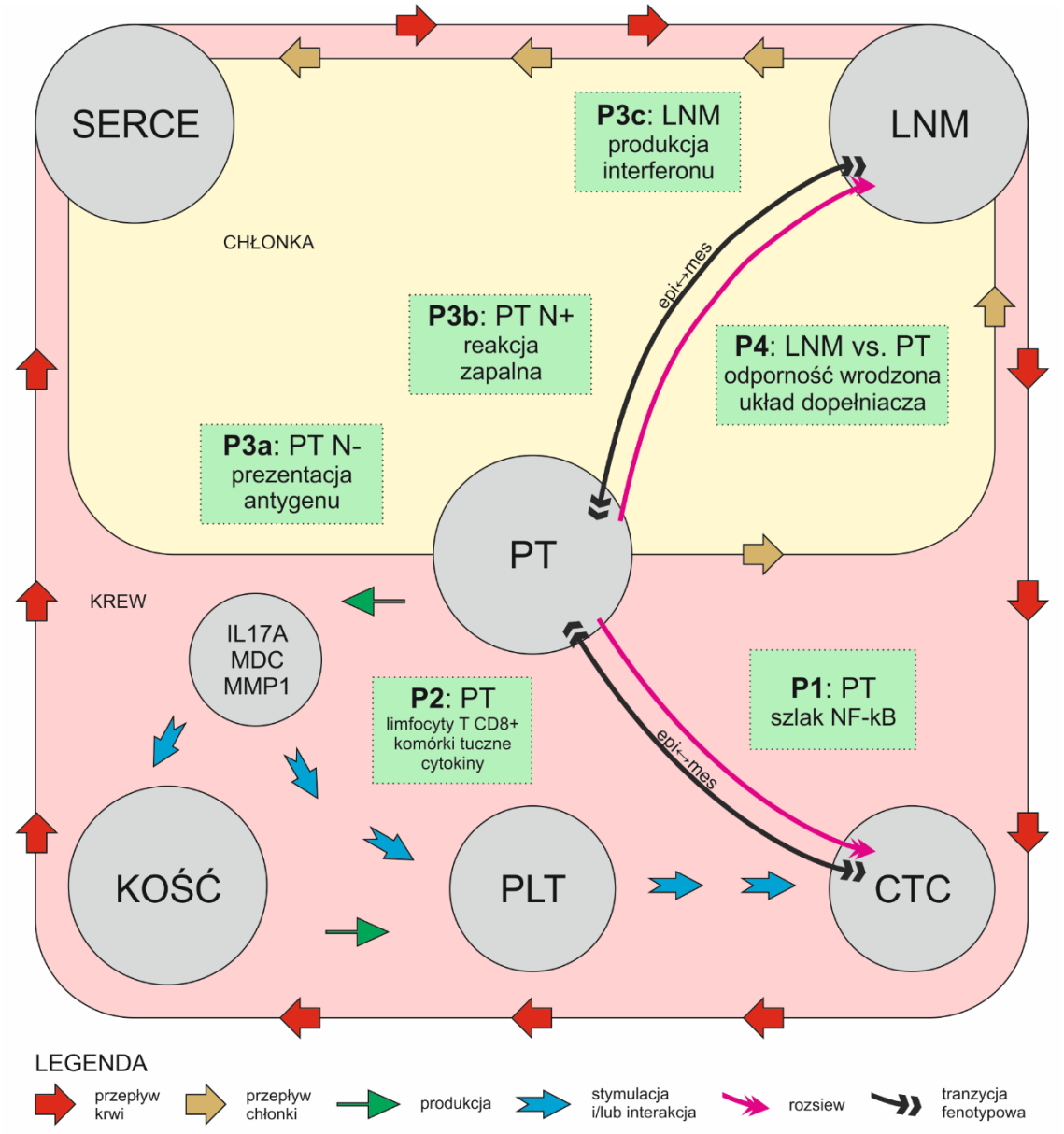
podścieliska, a konkretnie dwóch populacji komórek infiltrujących – komórek tucznych oraz limfocytów T CD8+, nasila produkcję cytokin takich jak IL17A [83–85], które stymulują trombopoezę [72] (**Rycina 3** w **[P2]**). Ponadto, zidentyfikowane cytokiny wytwarzane przez komórki podścieliska warunkują również pro-nowotworową aktywność płytek krwi (MMP1 [86]) oraz ich aktywację (MDC [CCL22] [86]). Proponowany przez nas systemowy mechanizm sprzyja rozsiewowi nowotworu, skutkując gorszą prognozą i krótszym czasem całkowitego przeżycia chorych o statusie hPC.

Kolejnym interesującym aspektem naszych badań był związek pomiędzy mikrośrodowiskiem i czynnikami około-immunologicznymi a aktywacją EMT w zależności od statusu rozsiewu nowotworu **[P3]**. Wiadome jest, że niektóre z cytokin i czynników wzrostu wytwarzanych przez komórki układu immunologicznego, które mogą wchodzić w skład nacieku infiltrującego guz, indukują EMT w komórkach nowotworowych. Dotyczy to m.in. IFNγ, TNFα, IL6 [87] czy TGFβ [88,89]. W naszej pracy wykazaliśmy zależne od fenotypu EMT różnice w zakresie immuno-transkryptomu guzów pierwotnych o statusie N- i N+ oraz ognisk przerzutowych w węzłach chłonnych. W każdym z trzech układów (PT N-, PT N+ oraz LNM) sygnatury związane z fenotypem komórek nowotworowych były odmienne, co dowodzi braku jednoznacznej zależności pomiędzy charakterystyką immunologiczną a statusem EMT w guzach pierwotnych piersi i ich przerzutach. Co istotne, również sam stopień aktywacji EMT w tych układach był zróżnicowany, co wskazuje, że wpływ fenotypu EMT komórek nowotworowych na zdolność rozsiewu jest zależny od szerszego kontekstu molekularnego.

Interesujące i biologicznie niejednoznaczne obserwacje poczyniliśmy również, analizując zmiany w obrębie immuno-transkryptomu komórek nowotworowych w ognisku przerzutowym względem ogniska pierwotnego **[P4]**. Jako pierwsi zaraportowaliśmy redukcję ekspresji genów związanych z funkcją układu dopełniacza w komórkach nowotworowych raka piersi, które zasiedliły węzeł chłonny, co potwierdziliśmy również na poziomie białka, wykonując oznaczenie ekspresji C3 – centralnego elementu układu dopełniacza. Podobnie jak w przypadku ścieżki sygnalizacyjnej NF-kB, zaobserwowane przez nas różnice w ekspresji dotyczyły różnych poziomów układu dopełniacza, poczynając od elementów wczesnych etapów jego aktywacji, przez regulatory, aż do efektorów. Układ dopełniacza odgrywa podwójną rolę w nowotworach, z jednej strony indukując odpowiedź układu immunologicznego przeciwko komórkom nowotworowym, z drugiej natomiast, generując przewlekły stan zapalny, który sprzyja rozwojowi guza [90]. Na ogół białka te są produkowane przez hepatocyty w wątrobie, skąd dystrybuowane są do całego organizmu za pośrednictwem krwi. W naszym badaniu mieliśmy do czynienia z lokalną ekspresją białek układu dopełniacza w komórkach nowotworowych, która budzi pewne kontrowersje i jak dotąd nie została szczegółowo opisana [91–93]. Podobnie rola układu dopełniacza w rozsiewie raka piersi pozostaje wciąż niedostatecznie poznana, a nasze doniesienie na temat utraty ekspresji białka C3 w przerzutach raka piersi do węzłów chłonnych jest pierwszym tego typu w literaturze. Kompleksowa analiza prognostycznego znaczenia ekspresji genów układu dopełniacza zaklasyfikowała rak piersi jako nowotwór, w którym układ dopełniacza pełni niejednoznaczną rolę [92], co niewątpliwie podnosi potrzebę dalszych badań w tym zakresie.

Podsumowując, badania ujęte w mojej rozprawie doktorskiej dostarczyły nowych informacji na temat udziału układu immunologicznego w rozsiewie raka piersi (**Rycina 1**). Wydaje się, że rola czynników około-immunologicznych w tym procesie jest niejednoznaczna, wielowymiarowa i zależna od kontekstu biologicznego. Na podstawie poczynionych obserwacji postulujemy, że szlak sygnalizacyjny NF-kB i układ dopełniacza stanowią potencjalnie kluczowe

dla przebiegu procesów rozsiewu punkty, stąd mogą stać się atrakcyjnym punktem uchwytu terapii celujących w formowanie przerzutów. Podobnie warte uwagi wydają się zależne od podścieliska procesy stymulujące trombopoezę. Wnioski płynące z przeprowadzonych badań na materiale klinicznym, jeśli potwierdzą się w dalszych badaniach w modelach *in vivo*, w przyszłości mogą dać podstawę do opracowania nowych rozwiązań terapeutycznych dedykowanych dla chorych z operacyjnym rakiem piersi.



Rycina 1. Graficzne podsumowanie wyników ujętych w rozprawie doktorskiej.

Guzy pierwotne rozsiewające krążące komórki nowotworowe o fenotypie mezenchymalnym wykazują podwyższoną ekspresję genów związanych ze ścieżką sygnalizacyjną NF-kB **[P1]**. Guzy pierwotne chorych z podwyższoną liczbą płytek krwi wykazują zwiększoną infiltrację limfocytów T CD8+ oraz komórek tucznych w fazie spoczynku oraz podwyższoną ekspresję genów kodujących cytokiny (IL17A [*IL17A*], MDC [*CCL22*] oraz MMP1 [*MMP1*]), które potencjalnie mogą skutkować nasileniem trombopoezy, aktywacji płytek oraz ich pro-nowotworowej aktywności **[P2]**. Mezenchymalne guzy pierwotne, które nie wytworzyły przerzutów do węzłów chłonnych, wykazują obniżoną ekspresję genów związanych z reakcją zapalną **[P3a]**. Mezenchymalne guzy pierwotne, które wytworzyły przerzuty do węzłów chłonnych, wykazują podwyższoną aktywność, potencjalnie również zwiększoną infiltrację, komórek prezentujących antygen **[P3b]**. Mezenchymalne przerzuty do węzłów chłonnych wykazują obniżoną ekspresję genów związanych z produkcją interferonu **[P3c]**. Przerzuty do węzłów chłonnych wykazują obniżoną ekspresję genów związanych z układem dopełniacza względem sparowanych guzów pierwotnych **[P4]**.

PT, guz pierwotny; LNM, przerzuty do węzłów chłonnych; N-, niezajęte węzły chłonne; N+, zajęte węzły chłonne; CTC, krążące komórki nowotworowe; PLT, płytki krwi; epi, epitelialny; mez, mezenchymalny.

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Publikacje wchodzące w skład rozprawy doktorskiej

Publikacja 1 [P1]

Popeda M, Stokowy T, Bednarz-Knoll N, Jurek A, Niemira M, Bielska A, Kretowski A, Kalinowski L, Szade J, Markiewicz A, Zaczek AJ

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NF-kappa B Signaling-Related Signatures Are Connected with the Mesenchymal Phenotype of Circulating Tumor Cells in Non-Metastatic Breast Cancer

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Abstract: The role of circulating tumor cells (CTCs), tumor microenvironment (TME), and the immune system in the formation of metastasis is evident, yet the details of their interactions remain unknown. This study aimed at exploring the immunotranscriptome of primary tumors associated with the status of CTCs in breast cancer (BCa) patients. The expression of 730 immune-related genes in formalin-fixed paraffin-embedded samples was analyzed using the multigenomic NanoString technology and correlated with the presence and the phenotype of CTCs. Upregulation of 37 genes and downregulation of 1 gene were observed in patients characterized by a mesenchymal phenotype of CTCs when compared to patients with epithelial CTCs. The upregulated genes were involved in NF-kappa B signaling and in the production of type I interferons. The clinical significance of the differentially expressed genes was evaluated using The Cancer Genome Atlas (TCGA) data of a breast invasive carcinoma (BRCA) cohort. Five of the upregulated genes—*PSMD7*, *C2*, *IFNAR1*, *CD84*, and *CYLD*—were independent prognostic factors in terms of overall and disease-free survival. To conclude, our data identify a group of genes that are upregulated in BCa patients with mesenchymal CTCs and reveal their prognostic potential, thus indicating that they merit further investigation.

Keywords: breast cancer; circulating tumor cells; epithelial–mesenchymal transition; NF-kappa B signaling; type I interferons; tumor microenvironment; immune-related transcriptome

1. Introduction

Distant metastases account for most of cancer-related deaths. Yet, fundamental questions regarding mechanisms that promote or inhibit the formation of metastasis still remain unanswered. It is evident that in breast cancer (BCa), tumor cell dissemination occurs already at early stages of the disease [1,2],

with circulating tumor cells (CTCs) being direct initiators of metastasis [3]. With current analytic methods, CTCs are detectable in the peripheral blood, and their presence is considered an adverse prognostic factor in numerous solid tumors, including BCa [4–11]. Tumor dissemination is known to be facilitated by the process of epithelial–mesenchymal transition (EMT) [12]. CTCs are shown to possess phenotypic plasticity that relates to their ability to display various EMT states in the circulation [13–15], with either the mesenchymal [16] or the intermediate [17] phenotype associated with increased tumor-initiating ability. The presence of mesenchymal CTCs is associated with disease progression and worse prognosis for metastatic BCa [18–20] and even operable breast cancer patients [21–23]. In fact, in metastatic BCa patients, the expression of mesenchymal markers is higher than in early-stage BCa patients [24,25] and correlates with lymph node involvement [26], suggesting that the EMT phenotype is directly related to the metastatic potential of CTCs.

The ability to evade the immune system is one of the hallmarks of cancer [27,28]. Tumor cells use multiple strategies to escape immune surveillance, mainly avoiding immune recognition and instigating an immunosuppressive microenvironment [29]. The interactions between cancer cells and their microenvironment (tumor microenvironment, TME) are involved in tumor dissemination. TME is assumed to modulate the capability of CTCs to evade the innate immune response and CTCs survival [30,31]. We showed that absence of ALDH1-positive stromal cells correlates with the presence of disseminated tumor cells (DTCs) in the bone marrow of BCa patients [32]. Nevertheless, the precise profile of immune-related factors influencing tumor dissemination, in particular the presence and phenotype of CTCs, is scarcely known. Therefore, the current study aimed at exploring the association between the immunotranscriptome of primary breast tumors and the status of CTCs. We hypothesized that the presence and the EMT phenotype of CTCs are connected with a specific immune-related gene signature of primary tumors. To verify this hypothesis, we evaluated the expression of 730 immune-related genes in primary tumors of BCa patients with well-described molecular and clinicopathological features, including CTCs presence and phenotype.

2. Results

2.1. Expression of Immune-Related Genes within Primary Tumours Correlated with the Phenotype of CTCs

To investigate the immune transcriptome associated with each phenotype of CTCs, we applied NanoString multigene expression analysis to samples of primary breast tumors. We observed that the mesenchymal phenotype of CTCs (n = 9) was associated with the upregulation of 37 genes and the downregulation of 1 gene in primary tumors (p-value ≤ 0.05, false discovery rate (FDR) ≤ 0.2) when compared to the epithelial phenotype of CTCs (n = 14) (Table 1, Figure S1; all results in Table S1). Due to the limited number of patients included in our study, we employed the conservative FDR method of multiple testing correction.

Our aim was also to explore the association between the expression of immune-related genes within the primary tumors and the overall presence of CTCs. We found no statistically significant differences between the primary tumors' immunotranscriptomes in relation to patients' CTC status (positive vs. negative).

Table 1. Genes up- and downregulated in primary tumors of patients with mesenchymal circulating tumor cells (CTCs) (compared to patients with epithelial CTCs).

Gene Symbol	Gene Name	FC	p-Value	FDR
LAIR2	Leukocyte-associated immunoglobulin-like receptor 2	3.19	0.007	0.144
FADD	Fas-associated via death domain	3.14	0.003	0.082
TLR7	Toll-like receptor 7	2.86	0.011	0.172
CCRL2	C–C motif chemokine receptor-like 2	2.80	0.003	0.087
PBK	PDZ-binding kinase	2.26	0.009	0.161
CD3EAP	CD3e molecule-associated protein	2.25	0.001	0.063
CD84	CD84 molecule	2.22	0.003	0.087

Table 1. *Cont.*

Gene Symbol	Gene Name	FC	*p*-Value	FDR
TNFSF13	TNF superfamily member 13	2.21	0.007	0.144
BIRC5	Baculoviral IAP repeat-containing 5	2.08	0.002	0.082
KLRC1	Killer cell lectin-like receptor C1	1.98	0.001	0.063
CD63	CD63 molecule	1.93	0.003	0.082
TNFRSF11A	TNF receptor superfamily member 11a	1.91	0.013	0.199
C2	Complement C2	1.85	0.001	0.069
IL1RAP	Interleukin 1 receptor accessory protein	1.79	0.011	0.172
TAPBP	TAP-binding protein	1.78	0.003	0.082
NUP107	nucleoporin 107	1.78	0.002	0.082
PSMD7	Proteasome 26S subunit, non-ATPase 7	1.72	<0.001	0.063
TICAM1	Toll-like receptor adaptor molecule 1	1.67	0.013	0.199
ICAM3	Intercellular adhesion molecule 3	1.63	0.009	0.161
IRF3	Interferon regulatory factor 3	1.59	<0.001	0.048
BCL10	BCL10 immune signaling adaptor	1.56	0.001	0.069
IKBKE	Inhibitor of nuclear factor kappa B kinase subunit epsilon	1.55	0.011	0.172
ELK1	ETS transcription factor ELK1	1.55	0.001	0.069
TRAF6	TNF receptor-associated factor 6	1.53	0.003	0.087
RELA	RELA proto-oncogene, NF-kB subunit	1.52	0.003	0.082
IKBKG	Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma	1.49	0.003	0.087
TBK1	TANK-binding kinase 1	1.46	0.001	0.063
STAT6	Signal transducer and activator of transcription 6	1.40	0.004	0.106
ATG10	Autophagy-related 10	1.40	0.007	0.144
CD74	CD74 molecule	1.38	0.009	0.161
ICOSLG	Inducible T cell costimulator ligand	1.38	0.002	0.082
FCGR2A	Fc fragment of IgG receptor IIa	1.37	0.007	0.144
PSMB10	Proteasome 20S subunit beta 10	1.36	0.011	0.172
CYLD	CYLD lysine 63 deubiquitinase	1.36	0.011	0.172
IFNAR1	Interferon alpha and beta receptor subunit 1	1.35	0.001	0.069
CCND3	Cyclin D3	1.30	0.002	0.082
MAP2K1	Mitogen-activated protein kinase kinase 1	1.25	<0.001	0.027
MERTK	MER proto-oncogene, tyrosine kinase	0.68	0.003	0.082

Fold change (FC) based on median normalized counts of the probe in each group; differences in median normalized counts between groups analyzed with Mann–Whitney U test (*p*-value) with Benjamini–Hochberg correction for multiple comparisons (false discovery rate, FDR); only statistically significant results are presented; gene names according to HUGO Gene Nomenclature.

2.2. The Mesenchymal Phenotype of CTCs Is Associated with the Upregulation of Genes Involved in NF-kappa B Signalling and Type I Interferons Production in Matched Primary Tumours

Here, we observed that multiple genes differentially expressed in patients with epithelial and mesenchymal CTC phenotypes (Table 1) play a role in the NF-kappa B signaling pathway. Consequently, we decided to interrogate this link more carefully. We applied the Functional Annotation Tool by DAVID 6.8 [33,34] to associate the selected genes with specific functional annotations. Genes upregulated in tumors with mesenchymal CTCs were generally involved in the activation and regulation of immune response (Table S2). Interestingly, 15 out of 37 upregulated genes (*FADD*, *TLR7*, *TNFRSF11A*, *IL1RAP*, *PSMD7*, *TICAM1*, *IRF3*, *BCL10*, *IKBKE*, *TRAF6*, *RELA*, *IKBKG*, *TBK1*, *PSMB10*, and *CYLD*) were implicated in the regulation of NF-kappa B signaling and activity (GO:0043122, GO:0051092, and GO:0038061). A literature search provided a number of links between other 11 differentially expressed genes (*CCRL2*, *PBK*, *TNFSF13*, *BIRC5*, *TAPBP*, *ELK1*, *STAT6*, *ATG10*, *IFNAR1*, *CCND3*, and *MAP2K1*) and the NF-kappa B pathway (top 12 genes depicted in Figure 1).

Analysis of Gene Ontology also revealed that nine of the upregulated genes regulate the production of type I interferons (GO:0032479; *TLR7*, *TICAM1*, *IRF3*, *IKBKE*, *RELA*, *TBK1*, *STAT6*, *CYLD*, and *IFNAR1*), with a particular role in the stimulation of interferon beta (GO:0032728; *TLR7*, *TICAM1*, *IRF3*, *TBK1*, and *IFNAR1*).

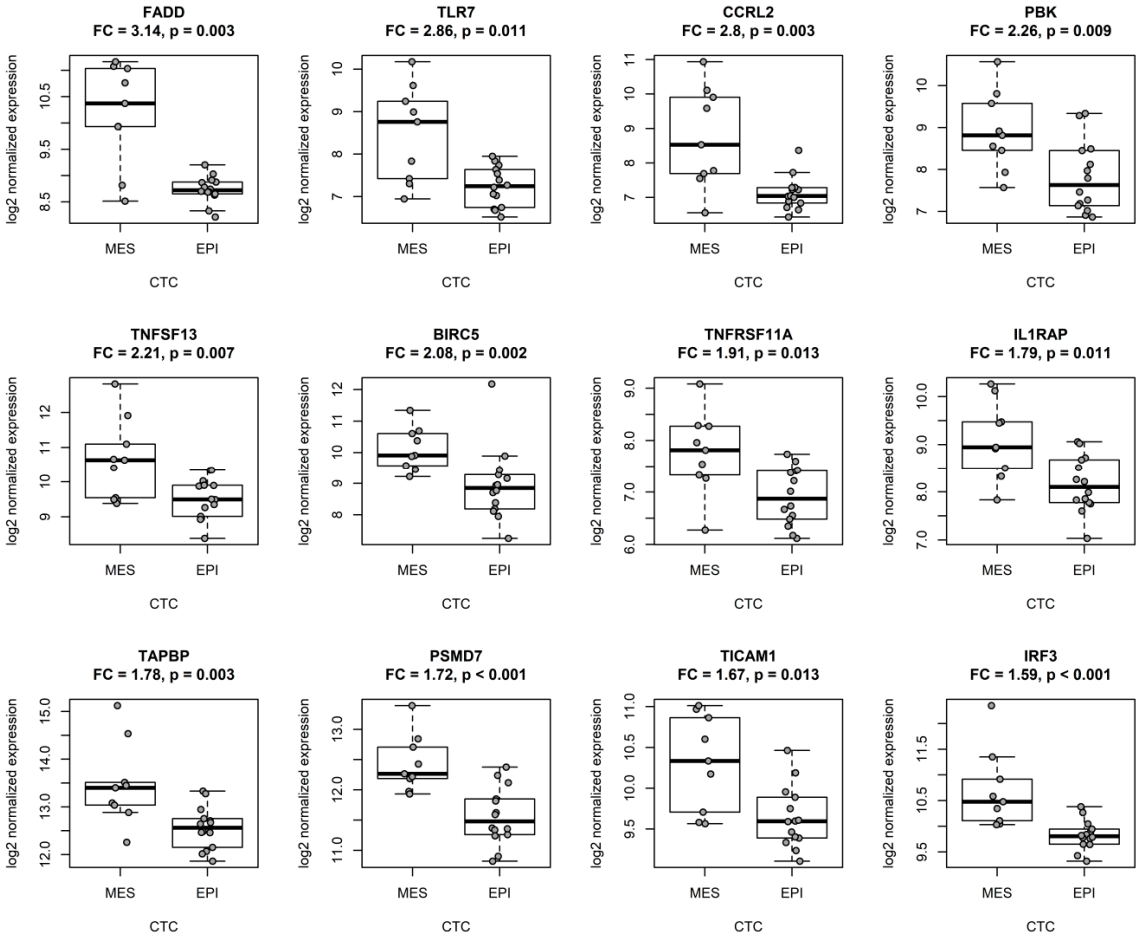

Figure 1. Genes implicated in NF-kappa B signaling were upregulated in primary tumors of breast cancer patients with mesenchymal CTCs (MES, $n = 9$) when compared to patients with epithelial CTCs (EPI, $n = 14$); the top 12 upregulated genes are presented. Gene expression depicted as number of counts of each probe and normalized to the four most stable reference genes (*ABCF1*, *EDC3*, *HDAC3*, and *CNOT4*); FC calculated on the basis of the median normalized counts of the probe in each group; differences in median normalized counts between groups analyzed with the Mann–Whitney U test (p); the bars correspond to the interquartile range (IQR), the whiskers cover 1.5 IQR from the median.

2.3. Immune-Related Genes Connected with the Mesenchymal Phenotype of CTCs Are Potent Negative Prognostic Factors in Breast Cancer

We have previously demonstrated that the mesenchymal phenotype of CTCs correlates with a poor prognosis in breast cancer patients [21]. Consequently, we decided to evaluate the prognostic significance of the immune-related genes that we found significantly up- or downregulated in primary tumors of BCa patients with mesenchymal CTCs. To this end, we turned to The Cancer Genome Atlas (TCGA) database and analyzed the available RNA-seq data on gene expression in a breast invasive carcinoma (BRCA) cohort ($n = 877$) [35,36]. Five out of 38 genes (*PSMD7*, *C2*, *IFNAR1*, *CD84*, and *CYLD*) associated with the mesenchymal phenotype of CTCs demonstrated a negative prognostic impact in the TCGA cohort. Moderate (higher than the first quartile, Q1 in Table S3) expression of *PSMD7* correlated with shorter overall survival (OS) in comparison to low expression of *PSMD7* in primary tumors (HR = 1.75, 95% CI: 1.08–2.82, $p = 0.022$; Figure 2A). A higher risk of recurrence was observed for tumors with moderate (higher than the first quartile, Q1 in Table S3) expression of *C2* (HR = 4.51, 95% CI: 1.36–14.96, $p = 0.014$; Figure 2B) and *IFNAR1* (HR = 2.68, 95% CI: 1.03–6.97, $p = 0.043$; Figure 2C) in comparison to tumors with low expression of these genes; high (higher than the third quartile, Q3 in Table S3) expression of *CD84* (HR = 2.48, 95% CI: 1.22–5.03, $p = 0.012$; Figure 2D) and *CYLD*

(HR = 2.20, 95% CI: 1.09–4.43, $p = 0.028$; Figure 2E) was also linked to shorter disease-free survival (DFS) in comparison to low expression of these genes in the primary tumors. Multivariate analysis including the clinical stage confirmed the significance of the aforementioned genes as independent prognostic factors (Table S3).

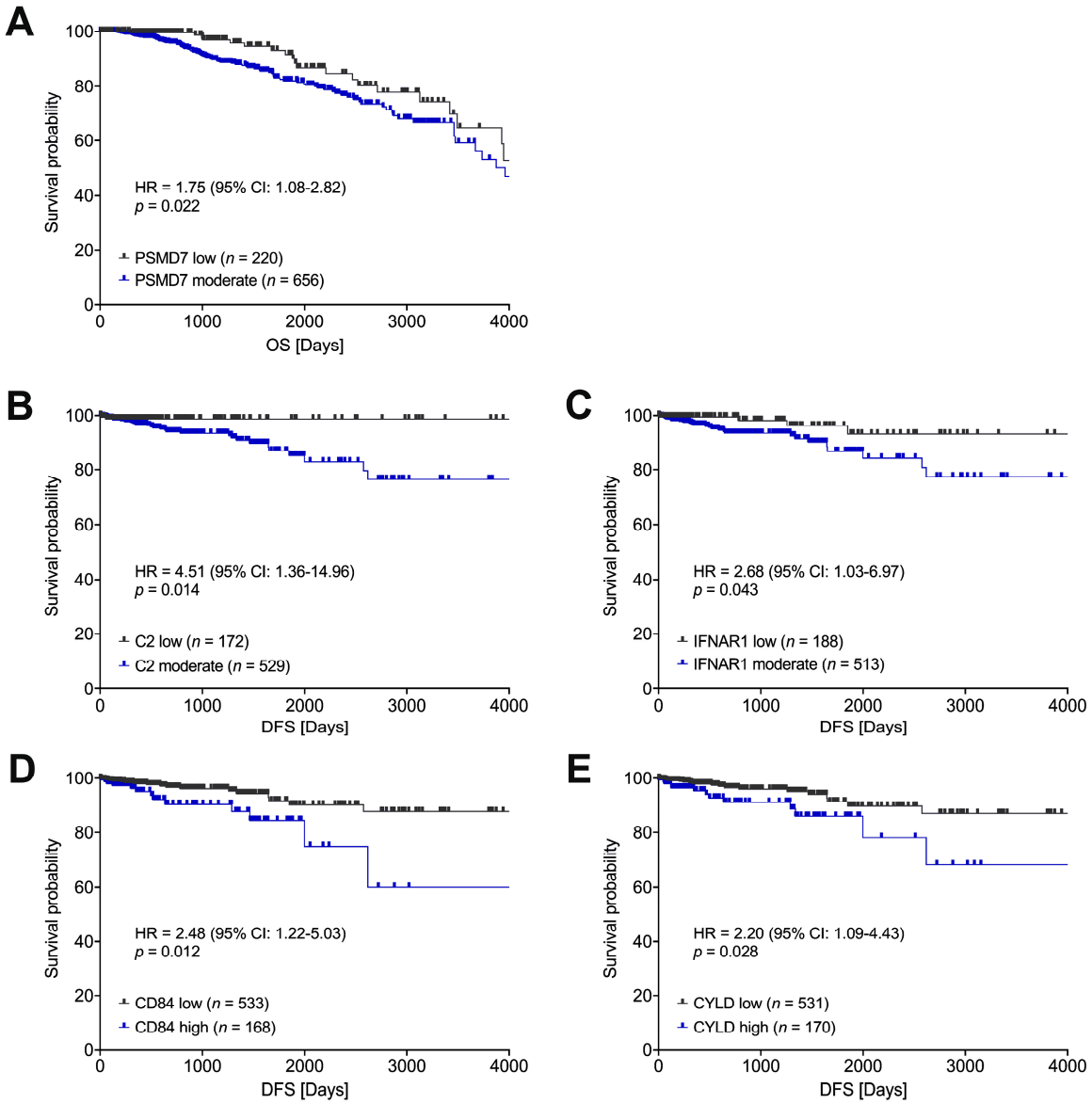

Figure 2. Genes associated with the mesenchymal phenotype of CTCs had a negative impact on survival (**A**) and recurrence (**B**–**E**) in patients in a BRCA cohort of The Cancer Genome Atlas (TCGA). Low/moderate status of gene expression relative to the first quartile (Q1); low/high status of gene expression relative to the third quartile (Q3); hazard ratios (HR) with 95% confidence intervals (95% CI) computed using Cox proportional hazards regression; OS: overall survival, DFS: disease-free survival.

3. Discussion

The knowledge about immune signatures related to tumor dissemination is still limited. Our current study aimed to identify the immunotranscriptomic profiles of primary tumors associated with the presence of CTCs and the CTCs phenotype in non-metastatic BCa patients.

Our data revealed that 38 genes were differentially expressed in the primary tumors of patients with mesenchymal CTCs when compared to patients with epithelial CTCs. Intriguingly, we did not observe any statistically significant difference between primary tumor transcriptomes when comparing

patients according to the presence or absence of CTCs in the circulation. The compared groups (CTC-positive and CTC-negative) were not biased in terms of any clinicopathological parameter (Table S4); hence, we believe that the observed difference in the activation of dissemination at BCa tumors may be cell context-dependent and definitely requires a more thorough analysis. On the other hand, our results demonstrate a substantial connection between the mesenchymal phenotype of CTCs and the NF-kappa B pathway. According to the NanoString gene expression assay and a literature search, 26 out of the 37 genes upregulated in mesenchymal-CTC patients in comparison to epithelial-CTC patients are implicated in NF-kappa B signaling at various levels of the transduction pathway (Figure 3A) and demonstrate a complex network of interactions at the protein level (Figure 3B). Importantly, the enrichment of NF-kappa B-related transcripts was consistently observed when we applied a stricter gene inclusion criteria and limited the analysis to a set of 330 genes with the highest expression (log2 mean count of a gene in all samples >9; Table S5).

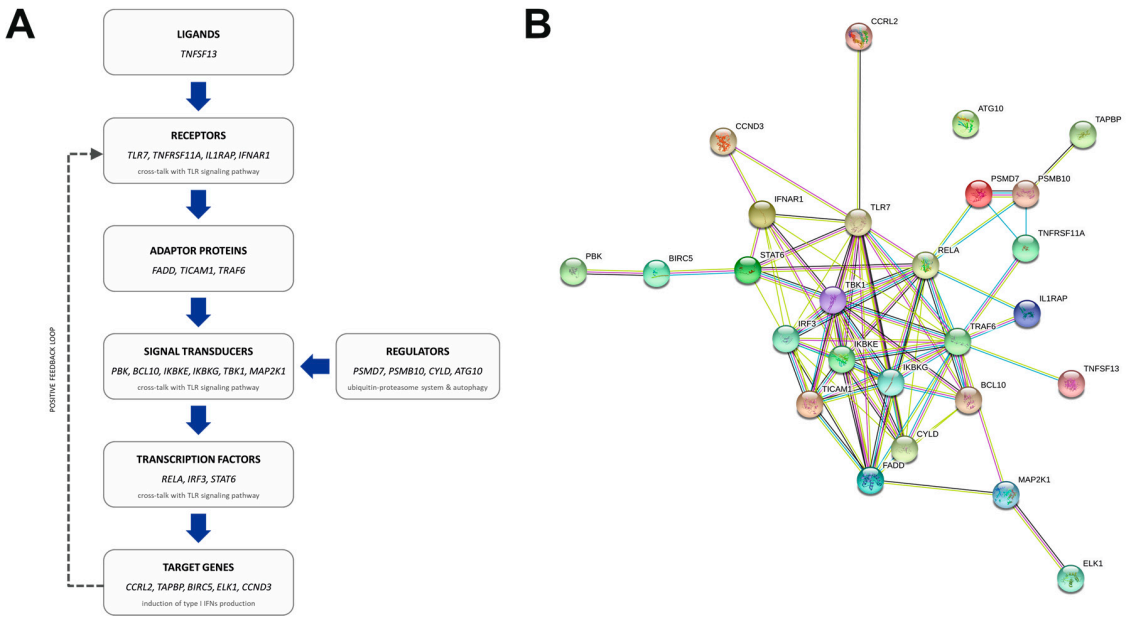

Figure 3. Genes upregulated in the primary tumors of patients with mesenchymal CTCs function at various levels of the NF-kappa B signaling pathway (**A**), within a complex network of interactions (**B**); image depicting a protein–protein association network, generated using the STRING tool; edge (line) coloring defines the type of interaction: blue—from curated databases, pink—experimentally determined, green—gene neighborhood, red—gene fusions, dark blue—gene co-occurrence, yellow—text mining, black—co-expression, violet—protein homology.

NF-kappa B signaling is a potent regulator of numerous vital physiological processes, including survival, inflammation, and immune responses [37]. The activation of the pathway is mediated by numeros receptors. Our enriched set (Table 1) includes genes encoding both specific ligands (APRIL (*TNFSF13*)) and receptors (*TLR7*, *TNFRSF11A*, *IL1RAP*, and *IFNAR1*), as well as universal adaptor proteins (*FADD*, *TICAM1*, and *TRAF6*) that facilitate the transduction of the signal from the receptors in the cell membrane to the effectors in the nucleus. Namely, we observed the upregulation of transducers involved in the canonical cascade (*BCL10* and *IKBKG*) as well as in Toll-like receptor-mediated activation of NF-kappa B signaling (*PBK*, *IKBKE*, and *TBK1*). Moreover, the enhanced expression of *MAP2K1* gene points to the possible role of ERK-mediated stimulation of NF-kappa B signaling in tumors with mesenchymal CTCs [38].

On the other hand, the activity of NF-kappa B is known to be regulated by the proteasome and ubiquitin-mediated proteolysis. Here, we report the upregulation of genes that are implicated in the ubiquitin-proteasome system (*PSMD7*, *PSMB10*, and *CYLD*) [39] and the autophagy cascade (*ATG10*) [40–42]. Eventually, we observed an increased expression of one the subunits of the NF-kappa

B transcription factor—p65 (*RELA*), as well as of two other co-operating transcription factors (*IRF3* and *STAT6*). The enhanced signaling resulted in the upregulation of five target genes—*CCRL2*, *TAPBP*, *BIRC5*, *ELK1*, and *CCND3*.

The NF-kappa B pathway is a well-known driver of EMT during both embryonic and tumor development [37]. In general, a constant stimulation of this pathway in cancer cells results in abnormal proliferation and differentiation, enhanced metastasis, and treatment resistance [43]. In breast cancer, NF-kappa B directly regulates the transcription of genes encoding EMT-inducing transcription factors [44]. In fact, the increased expression of NF-kappa B is a common feature of breast cancer cell lines and tissues, correlating with intensified activation of both the canonical and the non-canonical pathway [45–47]. What is more, several reports point to an interesting association between NF-kappa B and HER2 [47–49], with evidence for predominant NF-kappa B activation in ER−/HER2+ breast tumors [45,49].

Our data revealed another interesting pattern of enrichment, with upregulation of several NF-kappa B-related genes that are particularly involved in the positive regulation of type I interferon production (Table S2). The cross-talk between NF-kappa B and Toll-like receptors (TLR)-mediated signaling results in an increased pro-inflammatory response that is additionally enhanced in an autocrine and paracrine manner by a positive feedback loop (Figure 3A) [50,51].

Noteworthy, among the NF-kappa B-unrelated genes, we found markers of platelet activation (*CD63* and *CD84*) [52,53], which is in line with literature reports on the co-operation between platelets and CTCs in the induction of EMT and metastasis formation [54,55]. Of note, tumor dissemination may also be supported by other populations of cells within the intratumoral stroma. The elevated NF-kappa B activity may result from increased release of pro-inflammatory cytokines by macrophages at the tumor site [56–58]. In fact, NF-kappa B seems to be involved in the polarization of tumor-associated macrophages [57]. We have previously reported the negative prognostic significance of CTCs of mesenchymal phenotype in BCa patients [21]. Due to the low number of patients in this cohort, in the current study we analyzed the impact of genes linked with mesenchymal CTCs in TCGA BCa cohort. In fact, in TCGA data 5 out of the 38 genes of our interest were associated with worse prognosis (overall survival or risk of recurrence), namely, *PSMD7*, *C2*, *IFNAR1*, *CD84*, and *CYLD*. None of the corresponding proteins is currently included in the routine histopathology for breast tumor, thus they need to be validated at the protein level in a large cohort of patients in order to prove their clinical importance and diagnostic applicability.

4. Materials and Methods

4.1. Patients

The study group consisted of 35 breast cancer patients staged I–III, who had undergone surgical treatment at the Medical University Hospital in Gdansk between April 2011 and May 2013. The study was approved by the Ethical Committee of the Medical University of Gdansk (NKBBN 94/2017), and informed consent was collected from all participants. Patients were characterized by different clinicopathological parameters (Table S4), with particular focus on CTC status—negative (n = 12) or positive (n = 23)—and molecular phenotype of CTCs—epithelial (n = 14) or mesenchymal (n = 9)—as described previously [26].

4.2. RNA Extraction

RNA was extracted from formalin-fixed paraffin-embedded (FFPE) primary breast tumor samples (four 10 µm-thick, unstained FFPE sections per patient) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration and purity were determined using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with Agilent RNA 6000 Pico Kit (Agilent Technologies).

4.3. nCounter Gene Expression Assay

Extracted RNA (4 µl) was pre-amplified using the nCounter Low RNA Input Kit (NanoString Technologies, Seattle, WA, USA) with the dedicated Primer Pool covering the sequences of 730 immune-related genes included in the nCounter PanCancer Immune Profiling Panel (NanoString Technologies). Pre-amplified samples were analyzed using the NanoString nCounter Analysis System (NanoString Technologies) according to the manufacturer's procedures for hybridization, detection, and scanning.

4.4. Data Analysis

For each tumor sample analyzed with the NanoString technology, the background level was estimated using the mean plus 2 standard deviations of the counts of the negative control probes included in the assay. Data were normalized using the geometric mean of the positive controls included in the assay and 4 most stably expressed housekeeping genes included in the PanCancer Immune Profiling Panel—*ABCF1*, *EDC3*, *HDAC3*, and *CNOT4*—(expression stability assessed with NormFinder, SD range 173.5–228.4 counts). Background thresholding and normalization were performed using nSolver 4.0 software (NanoString Technologies).

Low-expression genes (log2 mean count of a gene in all samples <6) were excluded, leaving 584 genes for further analysis. Subsequently, the genes differentiating each CTC status were selected on the basis of fold change in comparison to the control; fold change was calculated on basis of the median normalized counts of the probe in each group. The following comparisons were performed: CTC-positive vs. CTC-negative; CTC-epithelial vs. CTC-negative; CTC-mesenchymal vs. CTC-negative; CTC-mesenchymal vs. CTC-epithelial. Genes with FC > 1 were considered upregulated; genes with FC < 1 were considered downregulated.

Data were analyzed using the R statistical computing environment (3.6.1) [59]. Differences in gene expression between groups were analyzed using the Mann–Whitney U test with Benjamini–Hochberg correction for multiple comparisons; p-values ≤ 0.05 and FDR values ≤ 0.2 were considered statistically significant.

For the differing genes, gene ontology was analyzed using the Functional Annotation Tool by DAVID Bioinformatics Resources 6.8 [33,34]. EASE Score, a modified Fisher exact p-value, was used to assess gene enrichment. Multiple testing was corrected using FDR correction.

For the NF-kappa B-related genes, a protein–protein association network was depicted using STRING v11 [60].

4.5. Survival Analysis in TCGA Cohort

RNA-seq (RNASeqV2, RSEM_ normalized) and clinical data of BRCA cohort were obtained from TCGA portal [35,36] (data status of 28 January, 2016). The group was limited to T1-3M0 patients, and records with missing clinical or expression values were excluded, leaving 877 out of 1098 BCa patients for the analysis. OS was defined according to the "days_to_death" variable for survival time and the "vital_status" variable for event; DFS was defined according to the "days_to_last_follow-up" variable for survival time and the "person_neoplasm_cancer_status" variable for event. For the genes of interest, low/moderate status of gene expression was determined according to the 1st quartile (Q1) cut-off, while low/high status of gene expression was determined according to the 3rd quartile (Q3) cut-off. For each gene, the expression status (low vs. moderate; low vs. high) was tested in both univariate and multivariate analyses including the clinical stage. Hazard ratios (HR) with 95% confidence intervals (95% CI) were computed using Cox proportional hazards regression using the R statistical computing environment (3.6.1) [59].

5. Conclusions

To summarize, this study points to the potential link between the expression of immune-related genes in cells within the primary tumor and the EMT state of circulating tumor cells. Increased NF-kappa B signaling-related signatures in the tumor mass might possibly promote EMT in CTCs, thus contributing to their more aggressive phenotype and worse patient prognosis. It merits further investigation whether such effect is due to the action of cancer cells or that of normal cells in the surrounding TME. The potential prognostic relevance of selected genes associated with mesenchymal CTCs is promising and deserves further validation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/12/1961/s1, Table S1: Results of gene expression analysis according to each CTC status or phenotype, Table S2: List of GO terms enriched in the genes upregulated in mesenchymal-CTC patients, Table S3: Results of multivariate survival analysis depending on the expression of genes up- and downregulated in mesenchymal-CTC patients; low/moderate status of gene expression relative to the 1st quartile (Q1); low/high status of gene expression relative to the 3rd quartile (Q3); hazard ratios (HR) with 95% confidence intervals (95% CI) computed using Cox proportional hazards regression; significant results are highlighted in green, Table S4: Clinicopathological characteristics of the patients, Table S5: List of genes upregulated in the primary tumors of patients with mesenchymal CTCs (compared to patients with epithelial CTCs), computed for a set of 330 high-expression genes (log2 mean count of a gene in all samples > 9), Figure S1: Expression of genes up- and downregulated in primary tumors of patients with mesenchymal CTCs (MES, $n = 9$) and patients with epithelial CTCs (EPI, $n = 14$); expression depicted as number of counts of each probe and normalized to the 4 most stable reference genes (*ABCF1*, *EDC3*, *HDAC3*, and *CNOT4*); fold change (FC) based on the median normalized counts of the probe in each group; differences in median normalized counts between groups analyzed with the Mann–Whitney U test (p); bars correspond to IQR, whiskers cover 1.5 IQR from the median.

Author Contributions: Conceptualization, T.S., M.P., A.M., N.B.-K., and A.J.Z.; methodology, T.S., A.J., M.P., M.N., A.M., and N.B.-K.; formal analysis, T.S., M.P., and A.J.Z.; investigation, A.M., N.B.-K., M.P., A.J., and A.B.; resources, J.S., A.K., and L.K.; data curation, M.P.; writing—original draft preparation, M.P., T.S., and A.J.Z.; writing—review and editing, all authors; visualization, T.S. and M.P.; supervision, T.S. and A.J.Z.; project administration, A.J.Z.; funding acquisition, A.J.Z.

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Materiały uzupełniające do Publikacji 1

Materiały uzupełniające dostępne na stronie internetowej czasopisma:

https://www.mdpi.com/2072-6694/11/12/1961/s1

Oświadczenia współautorów do Publikacji 1

Gdańsk, dnia 13 06 2022

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P1]

Popeda M, Stokowy T, Bednarz-Knoll N, Jurek A, Niemira M, Bielska A, Kretowski A, Kalinowski L, Szade J, Markiewicz A, Zaczek AJ

NF-kappa B Signaling-Related Signatures Are Connected with the Mesenchymal Phenotype of Circulating Tumor Cells in Non-Metastatic Breast Cancer

Cancers (Basel). 2019 Dec 6;11(12):1961. doi: 10.3390/cancers11121961

mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- opracowanie metodologii analizy immunotranskryptomu, w tym analizy bioinformatycznej;
- wykonanie analizy immunotranskryptomu (przygotowanie materiału tkankowego do analizy metodą nCounter, ekstrakcja RNA; analiza bioinformatyczna) oraz analizy przeżycia (dane z projektu TCGA); analizę statystyczną, opracowanie wyników i ich interpretację;
- zarządzanie danymi;
- przygotowanie manuskryptu;
- przygotowanie rycin i tabel;
- przygotowanie odpowiedzi na recenzję.

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Oświadczam, iż w pracy [P1]

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Cancers (Basel). 2019 Dec 6;11(12):1961. doi: 10.3390/cancers11121961

mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- udział w opracowaniu metodologii analizy immunotranskryptomu (ekstrakcja RNA);
- udział w interpretacji wyników analizy immunotranskryptomu i analizy przeżycia;
- udział w odpowiedzi na recenzję.

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P1]

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Cancers (Basel). 2019 Dec 6;11(12):1961. doi: 10.3390/cancers11121961

mój wkład obejmował:

- nadzór merytoryczny nad przygotowaniem materiału tkankowego do analizy immunotranskryptomu (analiza histopatologiczna).

(podpis)

Gdańsk, dnia 9/6/2022

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P1]

Popeda M, Stokowy T, Bednarz-Knoll N, Jurek A, Niemira M, Bielska A, Kretowski A, Kalinowski L, Szade J, Markiewicz A, Zaczek AJ

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Cancers (Basel). 2019 Dec 6;11(12):1961. doi: 10.3390/cancers11121961

mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- opracowanie metodologii izolacji i analizy CTC;
- udział w interpretacji wyników analizy immunotranskryptomu i analizy przeżycia;
- udział w odpowiedzi na recenzję.

Aleksandra Markiewicz
(podpis)

Gdańsk, dnia 13.06.2022

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P1]

Popeda M, Stokowy T, Bednarz-Knoll N, Jurek A, Niemira M, Bielska A, Kretowski A, Kalinowski L, Szade J, Markiewicz A, Zaczek AJ

NF-kappa B Signaling-Related Signatures Are Connected with the Mesenchymal Phenotype of Circulating Tumor Cells in Non-Metastatic Breast Cancer

Cancers (Basel). 2019 Dec 6;11(12):1961. doi: 10.3390/cancers11121961

mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- redakcję manuskryptu;
- udział w odpowiedzi na recenzję;
- nadzór merytoryczny nad badaniami;
- pozyskanie środków na badania i kierowanie projektem;
- zapewnienie zaplecza badawczego, w tym niezbędnej infrastruktury;
- koordynację działań projektowych, w tym pozyskania materiału klinicznego, danych klinicznych i histopatologicznych oraz analizy molekularnej.

(podpis)

Publikacja 2 [P2]

Bednarz-Knoll N, **Popęda M**, Kryczka T, Kozakiewicz B, Pogoda K, Szade J, Markiewicz A, Strzemecki D, Kalinowski L, Skokowski J, Liu J, Żaczek AJ

Higher platelet counts correlate to tumour progression and can be induced by intratumoural stroma in non-metastatic breast carcinomas

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British Journal of Cancer www.nature.com/bjc

ARTICLE

Molecular Diagnostics

Higher platelet counts correlate to tumour progression and can be induced by intratumoural stroma in non-metastatic breast carcinomas

Natalia Bednarz-Knoll[1✉], Marta Popęda[1], Tomasz Kryczka[2,3], Barbara Kozakiewicz[4,5], Katarzyna Pogoda[6], Jolanta Szade[7], Aleksandra Markiewicz[1], Damian Strzemecki[3,8], Leszek Kalinowski[9,10], Jarosław Skokowski[11], Jian Liu[12] and Anna J. Żaczek[1✉]

BACKGROUND: Platelets support tumour progression. However, their prognostic significance and relation to circulating tumour cells (CTCs) in operable breast cancer (BrCa) are still scarcely known and, thus, merit further investigation.
METHODS: Preoperative platelet counts (PCs) were compared with clinical data, CTCs, 65 serum cytokines and 770 immune-related transcripts obtained using the NanoString technology.
RESULTS: High normal PC (hPC; defined by the 75th centile cut-off) correlated with an increased number of lymph node metastases and mesenchymal CTCs in the 70 operable BrCa patients. Patients with hPC and CTC presence revealed the shortest overall survival compared to those with no CTC/any PC or even CTC/normal PC. Adverse prognostic impact of hPC was observed only in the luminal subtype, when 247 BrCa patients were analysed. hPC correlated with high content of intratumoural stroma, specifically its phenotype related to CD8+ T and resting mast cells, and an increased concentration of cytokines related to platelet activation or even production in bone marrow (i.e. APRIL, ENA78/CXCL5, HGF, IL16, IL17a, MDC/CCL22, MCP3, MMP1 and SCF).
CONCLUSIONS: Preoperative platelets evaluated alone and in combination with CTCs have prognostic potential in non-metastatic BrCa and define patients at the highest risk of disease progression, putatively benefiting from anti-platelet therapy.

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BACKGROUND

Platelets play a key role in regulating tumour cells' survival in the blood stream and metastasis efficiency [1, 2]. They can adhere to circulating tumour cells (CTCs), support their extravasation [3, 4], induce their proliferation [5] and even recruit and activate macrophages or neutrophils to induce tumour escape from natural killer cell lysis [6]. Putatively platelets can also initiate or at least maintain epithelial-mesenchymal transition (EMT) in CTCs, which might result in the generation of a (semi-) mesenchymal phenotype [7, 8] that is assumed to indicate higher metastatic potential of CTCs [9]. This phenomenon can influence CTC detection using standard methods, such as epithelial cell marker-based isolation and characterisation; thus, it can result in false-negative enumeration of CTCs [9]. Preoperative platelet counts (PCs) and platelet-to-lymphocyte ratios have been demonstrated to predict patient outcomes in certain tumour entities [10–12], including breast cancer and even its most aggressive triple-negative subtype [13–15]. Finally, the prevention or inhibition of platelet activation by antithrombotic drugs, such as aspirin, heparin, warfarin or novel nanoparticle cocktails, can result in improved patient outcomes and even attenuate the formation of the metastatic niche [16–19].

Platelets release small molecules, such as cytokines/chemokines, which, in turn, can directly or indirectly regulate CTCs and their fate. Cytokines/chemokines mediate the communication between cells and can be secreted by primary tumours, intratumoural stromal cells, immune cells or even different cellular components of homing organs or already existing distant (micro)metastasis. However, only a few studies have investigated cytokines/chemokines in relation to CTCs, including those on breast carcinoma [20–23]. The concentrations of cytokines/chemokines differ depending on the status and

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characteristics of CTCs [22, 23] and correlate with patient outcomes in different solid tumours, including breast cancer [24]. Some of them are considered therapeutic targets [25], whereas others (e.g. tumour growth factor beta (TGFβ) or platelet-derived growth factor) can mediate platelet regulation of the tumour cell phenotype [1, 8].

This study aims to investigate the clinical relevance of platelets and their potential interplay with CTCs in non-metastatic breast cancer. The PCs and selected serum cytokines/chemokines are correlated with the clinical outcome, presence and phenotype of CTCs and molecular features of primary tumours to reveal a potential network of interactions.

METHODS

Breast cancer patients

Cohort I: patients with CTC and primary tumour characteristics. Female breast cancer patients ($n = 108$) were treated in the Medical University Hospital in Gdańsk, Poland, during 2010-2013. All patients were staged I–III according to the American Joint Committee on Cancer staging manual version 7 [26]. Patients with co-morbidities or secondary cancer, undergoing neoadjuvant therapy, with lobular and mixed (i.e. lobular-ductal) breast cancer (characterised by innate loss of E-cadherin putatively falsifying the acquired EMT status) and those for whom preoperative blood count data were not available were excluded from this study. Due to fewer patients with PC beyond the normal range, the patients were limited to those with PC ranged between 150,000–400,000 platelets/μl ($n = 70$). Their blood counts and clinical parameters were documented (Supplementary Tables 1 and 2). Overall survival (OS) was defined as the time between the mastectomy and the patient's death. The last follow-up was completed in February 2016, and the median follow-up was conducted after 4.1 years (range 1.6–5.3 years).

Informed consent was collected from all study participants, and the permission to conduct this experiment was taken from the Bioethical Committee of the Medical University of Gdansk (NKEBN/30/2010, approved on the 17 March 2010).

Cohort II: extended cohort of patients with different age and molecular subtypes of breast cancer. Female non-metastatic breast cancer patients without previous and concurrent malignancies, staged I–III treated in the Medical University of Gdańsk ($n = 70$, Gdańsk, Poland) and Medical University of Warsaw ($n = 184$, Warsaw, Poland), were included in this cohort based on their preoperative PC within normal range ($n = 254$). Their clinical parameters, including age and molecular subtype were documented (Supplementary Table 3).

The study was conducted in accordance with the Helsinki Declaration of 1975, STROBE [27] and REMARK study recommendations [28].

Blood counts

Blood counts were performed in the routinely collected preoperative blood samples in the certified institution of each involved centre (i.e. Central Clinical Laboratory of the Medical University Hospital, Gdańsk, Poland and Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland) by using standard automated methods. In addition, lymphocyte-to-neutrophil, lymphocyte-to-monocyte, platelet-to-lymphocyte, eosinophil-to-lymphocyte, basophil-to-lymphocyte and eosinophil-to-basophil ratios were calculated. Different cut-offs were tested in order to divide patients in regard to their PC. As a final stratification system, in the presented study patients were dichotomised into groups with normal PC (nPC) and high normal PC (hPC) according to the 75th centile cut-off equal 289,000 platelets/ μl.

CTC isolation and characterisation

CTCs were isolated and enriched from preoperatively collected blood samples (at a volume of 5 ml) of Cohort I patients and phenotyped blindly to the clinico-pathological data for the gene expression of HER2/mammaglobin 1/cytokeratin 19/vimentin as previously described [26]. Briefly, the CTC-enriched fraction was isolated, and RNA was extracted, pre-amplified and quantified by the real-time polymerase chain reaction. MGB1+ and/or HER2+ samples identified as (i) CK19+/VIM− were classified as epithelial CTC-positive, whereas those identified as (ii) CK19−/VIM+ were classified as mesenchymal CTC-positive.

Cytokine/chemokine analysis

The blood samples (at a volume of 3.5 ml) for cytokine/chemokine analysis were obtained preoperatively from 36 Cohort I patients and characterised as CTC-negative (CTC−, $n = 24$), epithelial CTC-positive (epiCTC+, $n = 7$) and mesenchymal CTC-positive (mesCTC+, $n = 5$). The blood sera were fractionated through centrifugation at 1300–2000 × *g* for 10 min within 30 min after blood collection and stored at −80 °C until further analysis.

The concentrations of the serum cytokines/chemokines (i.e. APRIL, BAFF, BLC, bNGF, CD30, CD40-ligand, ENA78, Eotaxin, Eotaxin-2, Eotaxin-3, FGF-2, Fractalkine, G-CSF/CSF-3, GM-CSF, GRO-alpha/KC, HGF, IFN-alpha, IFN-gamma, IL-1alpha, IL-1beta, IL-10, IL-12p70, IL-13, IL-15, IL-16, IL-17A, I-18, IL-2, IL-2R, IL-20, IL-21, IL-22, IL-23, IL-27, IL-3, IL-31, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10, I-TAC, LIF, MCP-1, MCP-2, MCP-3, M-CSF, MDC/CCL22, MIF, MIG, MIP-1alpha, MIP-1beta, MIP-2alpha, MMP1, SCF, SDF-1alpha, TNF-alpha, TNF-beta, TNF-RII, TRAIL, TSLP, Tweak and VEGF-A) were measured using a commercially available magnetic microbead-based 65-Plex Human ProcartaPlex™ Panel and a Bio-Plex® 200 system based on Luminex xMAP technology (Bio-Rad) according to the manufacturer's instructions. The analysed blood sera were blinded to the experimenter, diluted in 1:4 ratio and measured in duplicate. The cytokine/chemokine concentrations were calculated within the range of the obtained standard curves at seven descending concentration points (experimental range 0.82–387,992 pg/ml, depending on the cytokine/chemokine).

Primary tumour characterisation

The epithelial or mesenchymal phenotype of the primary tumour of the Cohort I patients was determined immunohistochemically by pathologist on the basis of E-cadherin, N-cadherin and vimentin expressions assessed in tumour cells, as previously described [26]. Briefly, an epithelial phenotype was determined in tumours characterised by the presence of E-cadherin and absence of both N-cadherin and vimentin, whereas a mesenchymal phenotype was determined in tumours characterised by E-cadherin loss or the presence of N-cadherin or vimentin [26].

The intratumoural stromal content within the primary tumour was evaluated using tissue microarrays stained with haematoxylin and eosin. Each tumour was investigated in five fragments (single tissue cores), each having a diameter of 1 mm. The stromal content was defined by the pathologist as the percent area of non-tumour components in each individually examined fragment of tumour. The highest assessed stromal content among the five examined tumor fragments was assigned to a patient and grouped according to the 75th centile (equal to 80%) in the groups with low or high stromal content.

Profiling of immune cells infiltrating primary tumours

The abundance of 22 leukocyte subsets in primary tumours was estimated for 32 Cohort I patients ($n = 32$, luminal BrCa) by using the CIBERSORTx digital cytometry algorithm [29]. In brief, the previously obtained immune-related transcriptome generated using the nCounter PanCancer Immune Profiling Panel (NanoString technology; data available at NCBI GEO under accession number GSE180186) [30] was subjected to an absolute mode analysis with a leukocyte gene signature matrix (LM22) and B-mode (bulk mode) batch correction following the CIBERSORTx manual (correlation coefficients of the analysed samples ranged 0.567–0.854, all having $p < 0.001$). The resulting absolute proportions of the leukocyte subsets were compared according to the patient's platelet count status (nPC vs. hPC). In addition, median-based fold changes (FCs) between those groups were evaluated. Differences were estimated using the Mann–Whitney test, with $p < 0.05$ considered statistically significant.

Statistics

All outcomes were documented and analysed statistically using SPSS ver. 25 licensed for the University of Gdańsk. The differences in the distribution of clinical parameters between the patients characterised by nPC and hPC were analysed using the chi-squared test or Fisher's exact test. The relationships between blood counts or cytokine/chemokine concentrations (presented as continuous values) and (i) CTC presence/phenotype, (ii) clinical parameters and (iii) molecular signatures of tumours or their stroma were analysed by conducting the Mann–Whitney or Kruskal–Wallis test. In addition, the outcomes for blood counts and immunohistochemically assessed molecular signatures of the tumour and stroma were classified into negative and positive groups according to the cut-offs equal to their 75th centile values. The associations among CTCs, platelet status and time to OS were evaluated using the log-rank test and Kaplan–Meier plot in R

statistical environment [31]. To estimate the hazard risk, a Cox-hazard-potential regression analysis (CI 95%) was conducted. All results with $p < 0.05$ were considered statistically significant. Cases with missing data were excluded from the analysis.

RESULTS

High normal platelet counts correlate with worse clinical outcome and mesenchymal phenotype of CTCs

The blood count was available for 75 of the 108 previously described [26] non-metastatic breast cancer patients, including a few patients with individual blood cell-type counts beyond the normal ranges. The cut-off at the 75th centile, equal to 289,000 platelets/µl, was used to define patients with nPC and hPC. Only three patients had platelet counts above the upper cut-off of the normal range (150,000–400,000 platelets/µl), which was not sufficient to perform reliable statistical analysis of patients with clinically defined thrombocytosis. Therefore, we narrowed the current analysis to patients within the normal range of preoperative platelet counts. Consequently, 70 breast cancer patients (Supplementary Table 1) with platelets within the normal range (Supplementary Table 2) were selected to study platelets' association with the OS and presence and phenotype of the CTC. The mean platelet count in this cohort was 257,728 platelets/µl (±SD51,257; range 160,000–390,000 platelets/µl). The other blood counts were defined beyond the normal range for some individual patients (Supplementary Table 2); however, as none of the patients were disqualified from surgery, we did not exclude any further patient from the study cohort. Note that all results presented in this study did not differ if the statistical analysis included three patients with platelet counts above 400,000 platelets/µl in the patient group with high PC (data not shown).

hPC found in 15 (21%) of 70 patients correlated with a higher tumour (T) status ($p = 0.001$, Fig. 1a, Supplementary Table 3), higher grading ($p = 0.032$, Fig. 1b, Supplementary Table 4) and the mesenchymal phenotype of CTCs ($p = 0.006$, Fig. 1c, Supplementary Table 4). PC was higher in CTC-positive patients but did not reach statistical significance (Mann–Whitney test, $p = 0.125$, Supplementary Fig. 1). hPC was also associated with a higher number of lymph node metastases in lymph node positive (N1) patients ($n = 38$, Mann–Whitney test, $p = 0.011$, Fig. 1d).

The presence of CTC(s) ($p = 0.005$, Fig. 1e), particularly their mesenchymal phenotype ($p = 0.011$, data not shown), correlated with the worse clinical outcome in the selected cohort of patients ($n = 70$), as described previously [26]. The hPC correlated with a shorter OS (Kaplan–Meier plot, log-rank test, $p < 0.0001$, Fig. 1f; Cox-hazard-potential regression analysis, $p = 0.001$; HR = 9.635, CI 95% 2.396–38.742, Table 1). The patients with both CTC-positivity and hPC were characterised by a shorter OS ($p < 0.001$) compared to those with (i) no CTC and any PC (Cox-hazard-potential regression analysis, $p = 0.001$; HR = 9.635, CI 95% 2.396–38.742) or even (ii) CTCs and nPC (Cox-hazard-potential regression analysis, $p = 0.078$; HR = 4.634, CI 95% 0.843–25.468, Fig. 1g). Of note, if patients were stratified according to their PC and CTC phenotype (Supplementary Fig. 2A), hPC/mes CTC status was the strong predictor of a shorter time to death when compared to nPC/no CTC (Supplementary Fig. 2B, Kaplan–Meier plot, log-rank analysis, $p < 0.001$) or even all other patients (Supplementary Fig. 2C, Kaplan–Meier plot, log-rank analysis, $p = 0.002$).

hPC showed the highest trend for association with the risk for death, however it failed to reach statistical significance in the multivariate analysis, including CTC, platelets, and T status ($p = 0.059$; HR = 4.722, CI 95% 0.940–23.6723, Table 1). When combined PC/CTC status was analyzed, in the univariate analysis, hPC/CTC status was associated with high risk of death (Cox-hazard-potential regression analysis, $p = 0.001$; HR 9.452, CI 95% 2.532–35.282). However, in the multivariate analysis, hPC/CTC did not reach statistical significance to be an independent prognostic marker (Cox-hazard-potential regression analysis, $p = 0.262$; HR 3.031, CI 95% 0.437–21.009, Supplementary Table 5).

To investigate the association between platelet count and OS in the context of other clinical parameters, including age and different molecular subtypes of breast cancer, we extended Cohort I by the dataset containing preoperative PCs within the normal range from non-metastatic breast cancer patients treated in the Medical University of Warsaw ($n = 184$, Warsaw, Poland) resulting in the Cohort II ($n = 254$, Supplementary Table 3). In this Cohort, PC did not correlate with any clinico-pathological parameter (Supplementary Table 6), whereas in the older patients and patients with luminal breast cancer it indicated worse outcome. Within five years after the surgery, the hPC correlated with worse OS in the sub-cohorts of all luminal cancers (i.e. luminal A + luminal B; $n = 151$, $p = 0.013$, Fig. 1h; Cox regression analysis $p = 0.021$, HR 3.626, CI 95% 1.216–10.817). However, it did not occur to be independent prognostic marker in multivariate analysis (Supplementary Table 7). Interestingly, in Her2-positive and triple-negative subtype of breast cancer, nPC was observed to correlate with worse prognosis but did not reach the statistical significance (Supplementary Fig. 3). Adverse effect of hPC on 5-years OS was also observed in the patients who were ≥50 ($n = 92$, Kaplan–Meier plot, log-rank test, $p = 0.010$, Supplementary Fig. 4), but was not confirmed in the multivariate analysis (Supplementary Table 8).

High normal platelet counts correlate with cytokine/chemokine profile in terms of their aggregation, activation and/or production

To identify the potential communication routes between platelets and CTCs or platelets and primary tumour or other blood cells, a broad panel of different signalling molecules (i.e. cytokines/chemokines) was screened in the sera of the selected patients.

Thirty (46%) of the 65 analysed cytokines/chemokines were detected in the blood sera of at least 4 (10%) out of the 36 examined patients (concentration range 1.17–34919 pg/µl, depending on the cytokine/chemokine concentration).

The hPC correlated with increased levels of APRIL (a proliferation-inducing ligand, also known as tumour necrosis factor superfamily member 13, TNFSF13, $p = 0.037$), epithelial-neutrophil activating peptide (ENA78/CXCL5, $p = 0.022$), hepatocyte growth factor (HGF, $p = 0.029$), interleukin 16 (IL16, $p = 0.010$), interleukin 17a (IL17a, $p = 0.033$), monocyte-chemotactic protein 3 (MCP3, $p = 0.037$), macrophage-derived chemokine (MDC/CCL22, $p = 0.005$), matrix metalloproteinase 1 (MMP1, $p = 0.054$) and stem cell factor (SCF, $p = 0.014$) (Fig. 2a). When the cytokine/chemokine concentrations were compared with the platelet status combined with CTC status, ENA78/CXCL5 and IL17a were significantly increased in the patients characterised by the presence of both CTC and hPC in contrast to those with no CTC/any PC or CTC/nPC ($p = 0.029$ and $p = 0.015$, respectively; data not shown).

High normal platelet counts correlate with intratumoural resting mast cells and CD8+ T-cell phenotype

Some of the selected cytokines/chemokines are known to be produced by platelets in their alpha-granules (e.g. ENA78/CXCL5 and MMP1 [1, 32] and putatively APRIL [33]). Other cytokines/chemokines might be secreted by other components (e.g. tumour cells, intratumoural stromal cells or other blood cells). In the current study, all cytokines/chemokines that were increased in the sera of the patients with hPC were tested against clinical data, available features of primary tumour and blood count (data not shown). Apart from the correlations to hPC, IL17a correlated with a high stromal content (defined by the cut-off at the 75th centile and equal to 80% stroma within the tumour fragment; $p = 0.031$;

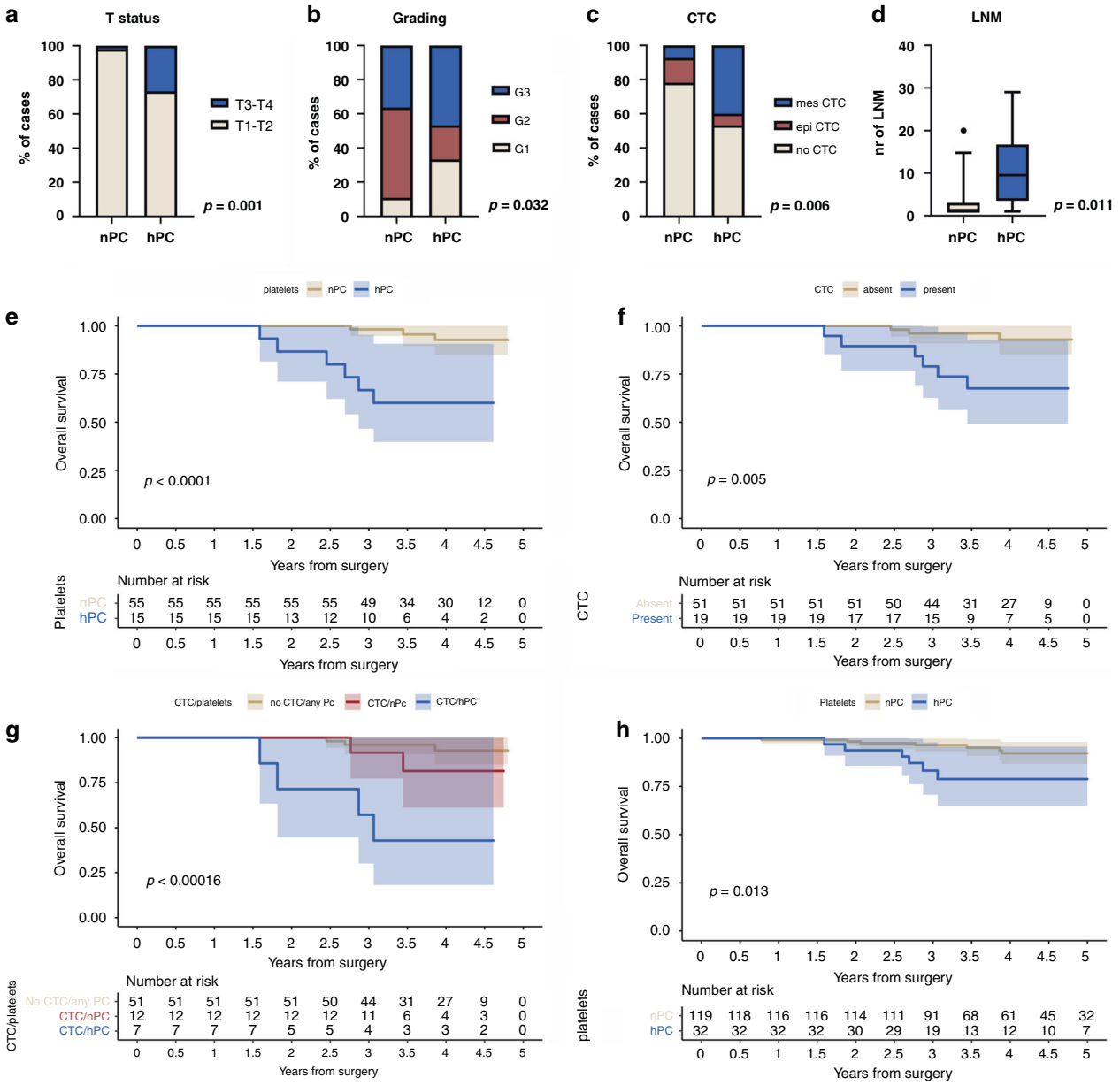


Fig. 1 Relationship between platelets and clinical data in breast cancer. Relationship between hPC and higher T status (**a**), grading (**b**), CTC phenotype ($n = 70$) (**c**) and higher number of LNMs ($n = 38$, lymph node positive (N1) patients) (**d**). Survival analysis of breast cancer patients ($n = 70$) in the context of platelet counts (**e**), CTC presence (**f**) and combined status of platelet count and CTC (**g**). Survival analysis of platelet count in patients with luminal breast cancer ($n = 151$; panel **h**). CTC – circulating tumour cell, epi CTC – CTC of epithelial phenotype, mes CTC – CTC of mesenchymal phenotype, nPC – normal platelet count, hPC – high normal platelet count, LNM – lymph node metastasis, OS – overall survival.

Fig. 2b), whereas MDC/CCL22 correlated with a higher number of eosinophils ($p = 0.005$; data not shown). hPC was also observed in patients with a high intratumoural stromal content ($p = 0.026$, Fig. 2b, c). Therefore, additional analysis aiming to profile hPC-related stromal components and the selected cytokine/chemokine profile in these patients was performed. Interestingly, when 770 gene expression outcomes obtained using the NanoString technology in 32 primary tumours (for which both platelets and NanoString data were available) were analysed using a digital cytometry algorithm CIBERSORTx [29], hPC correlated with the increased abundance of both CD8+ T cells (FC = 1.88, $p = 0.026$) and resting mast cells (FC = 1.54, $p = 0.041$) (Fig. 2d).

When comparing patients with transcriptomic signatures indicating high and low concentrations of resting mast cells in primary tumours (using CIBERSORTx absolute mode and upper-quartile cut-off), a significant increase in the serum levels of IL17a ($p = 0.033$) was observed in the group with estimated high mast cell infiltration (Supplementary Fig. 5A). In addition, a comparison of signatures indicating the presence of CD8+ T cells revealed that the MMP1 ($p = 0.01$) and MDC/CCL22 ($p = 0.01$) levels were increased in tumours with high CD8+ T-cell infiltration (Supplementary Fig. 5B and C, respectively).

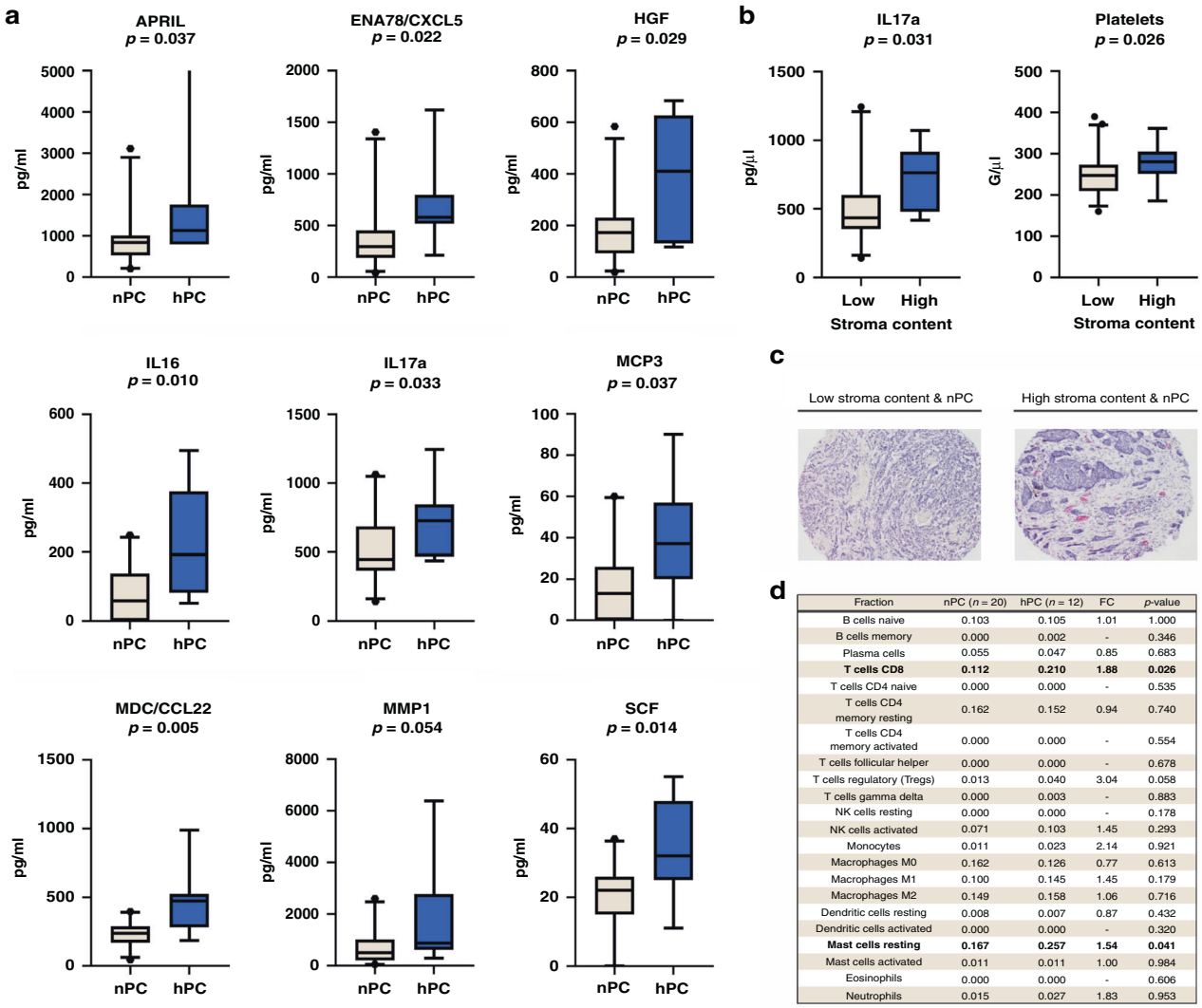
DISCUSSION

Platelets are considered a primary element of the blood microenvironment modulating the efficacy of the metastatic cascade. This paper demonstrates the prognostic value of platelets

Table 1. Impact of platelets on OS of breast cancer patients in multivariate analysis.

Parameter	Group	Univariate			Multivariate		
		p-value	HR	CI 95%	*p*-value	HR	CI 95%
Age	≥50 vs. <50	0.984	0.984	0.204–4.741	–	–	–
T status	T3-4 vs. T1-2	**<0.001**	**1.123**	**1.057–1.193**	0.197	1.052	0.974–1.137
N status	N1 vs. N0	0.099	3.751	0.778–18.085	–	–	–
Tumour grade	G3 vs. G1-2	0.723	0.974	0.842–1.127	–	–	–
Hormone receptor status	neg vs. pos	0.501	2.042	0.255–16.335	–	–	–
Her2 status	pos vs. neg	0.892	0.897	0.186–4.320	–	–	–
Platelet status	hPC vs. nPC	**0.001**	**9.635**	**2.396–38.742**	0.059	4.722	0.940–23.723
CTC status	pos vs. neg	**0.013**	**5.783**	**1.445–23.138**	0.298	2.397	0.463–12.425

Statistically significant results are presented in bold. Platelet range: 150,000–400,000, pts $n = 69$.
HR hazard ratio, *CI* confidence interval, *hPC* high normal platelet count, *nPC* normal platelet count, *CTC* circulating tumour cells, *neg* negative, *pos* positive.



Fraction	nPC ($n = 20$)	hPC ($n = 12$)	FC	*p*-value
B cells naive	0.103	0.105	1.01	1.000
B cells memory	0.000	0.002	-	0.346
Plasma cells	0.055	0.047	0.85	0.683
T cells CD8	**0.112**	**0.210**	**1.88**	**0.026**
T cells CD4 naive	0.000	0.000	-	0.535
T cells CD4 memory resting	0.162	0.152	0.94	0.740
T cells CD4 memory activated	0.000	0.000	-	0.554
T cells follicular helper	0.000	0.000	-	0.678
T cells regulatory (Tregs)	0.013	0.040	3.04	0.058
T cells gamma delta	0.000	0.003	-	0.883
NK cells resting	0.000	0.000	-	0.178
NK cells activated	0.071	0.103	1.45	0.293
Monocytes	0.011	0.023	2.14	0.921
Macrophages M0	0.162	0.126	0.77	0.613
Macrophages M1	0.100	0.145	1.45	0.179
Macrophages M2	0.149	0.158	1.06	0.716
Dendritic cells resting	0.008	0.007	0.87	0.432
Dendritic cells activated	0.000	0.000	-	0.320
Mast cells resting	**0.167**	**0.257**	**1.54**	**0.041**
Mast cells activated	0.011	0.011	1.00	0.984
Eosinophils	0.000	0.000	-	0.606
Neutrophils	0.015	0.027	1.83	0.953

Fig. 2 Relationship between platelets and molecular data in breast cancer patients. Relationship between cytokine/chemokine levels and platelet status in non-metastatic breast cancer patients with nPC ($n = 23$) and hPC ($n = 7$) (**a**). Relationship of intratumoural stroma with higher IL17a concentration (20 patients with low stromal content vs. 10 patients with high stromal content) and higher platelet counts (53 patients with low stromal content vs. 17 patients with high stromal content) (**b**). Representative microscope images of low and high stromal contents in tumours of patients with nPC and hPC, haematoxylin and eosin staining, magnification ×100 (**c**). Leukocyte subset abundance in primary tumours according to platelet count status (CIBERSORTx analysis; for each fraction, median absolute scores in compared groups are presented; panel **d**). Significant correlations are presented in bold. CTC indicates circulating tumour cells, FC fold change, nPC normal platelet count, hPC high normal platelet count.

in non-metastatic breast cancer. This is the first study that shows the correlation of hPC with mesenchymal CTCs and worse clinical outcome.

In this study, the platelet counts were restricted to the standard normal range (i.e. 150,000–400,000 platelets/µl), as only three patients in the CTC cohort were characterised as having higher platelet counts related to thrombocytosis, which is commonly associated with adverse outcomes. Thus, a reliable statistical analysis of the correlation of thrombocytosis or three groups of patients (i.e. patients with nPC, hPC and thrombocytosis) with CTC or clinical outcome could not be performed. However, the presented outcomes remained true even if the three patients with defined thrombocytosis were included in the subgroup of patients with high platelet counts (data not shown). In concordance with our data, Ankus et al. observed that hPC might be associated with a higher risk of cancer occurrence, including breast cancer [34], whereas in head and neck squamous cell carcinoma, hPC was correlated with worse OS [35]. In the study of a large cohort of 40,987 healthy inhabitants of seven Italian areas, the normal range of platelets was proposed to be revised in the context of age, as the range of platelet counts was significantly diminished in the proband groups above 64 years of age [36]. This observation could potentially explain why in our cohorts of patients predominantly characterised by older age, the effect of hPC classified with a relatively low cut-off (i.e. 289,000 platelets/µl) was not considered as classical thrombocytosis.

The study results corroborate the hypothesis that preoperative hPC, as well as the coincidence of CTCs, in particular mes CTC, and hPC, reflects an advanced stage of disease and poor prognosis and can have a critical impact on the metastatic process. This observation is consistent with the preclinical data obtained by Labelle et al. for a mouse model [7, 8], where the platelets supported the metastatic process. Here, the higher PC defined even within the standard normal range correlated with the worse clinical outcome and occurred more frequently in blood samples enriched by CTCs characterised by the mesenchymal phenotype, which represent a particularly aggressive type of CTCs [9]. The association of hPC with mesenchymal but not epithelial CTCs is found based on very few patients and, thus, needs further investigation. Nevertheless, hPC seems to enhance the adverse impact of CTCs on patients' survival: patients with CTCs, in particular mes CTC, and hPC exhibit a worse prognosis more frequently than those with no CTCs and any platelet count or even CTCs and nPC. This finding is supported by the fact that hPC is the only factor that impacts OS in multivariate analysis, including the statuses of T, platelets and CTCs. Although this correlation is only borderline (which might be biased by the low number of patients included in this analysis), it indicates the postulated link between hPC and tumour progression. Finally, in our study, the platelets correlate with a higher number of established metastases in lymph nodes. Tumour dissemination to lymph nodes is considered to occur predominantly through lymphatic vessels. The correlation observed in this study suggests that vascular vessels form an alternative route and that metastatic spread is more efficient in a microenvironment abundant in platelets.

Intriguingly, in the larger cohort of patients ($n = 254$), hPC did not correlate to age and molecular subtype, but indicated worse clinical outcome both in older patients and luminal cancers. It might be speculated that those differences result from hormonal changes occurring both during aging and luminal cancers treatment as estrogen receptor is expressed on platelets [37, 38]. This observation, including the underlying mechanisms, should be carefully exploited for a large cohort of patients.

The relation between platelets and CTCs is being extensively studied. A series of recent data have suggested that platelets interact with CTCs, facilitating their later extravasation [1, 3, 4] and tumour cell escape from the immune response [6]. Moreover, CTCs can undergo EMT in the blood stream within a short transit time upon direct contact with platelets under platelet-derived TGFβ stimulation. In our study, hPC is associated with the mesenchymal phenotype of CTCs but not with the phenotype of tumour cells examined within primary tumours (Supplementary Table 4). However, the number of patients for whom the matched samples of primary tumour and blood are analysed is too small to observe whether the change in the phenotype of tumour cells indeed occurs in the blood stream and is platelet-dependent (10 pairs of primary tumour-blood samples with defined tumour cell phenotype; data not shown). Therefore, further studies, particularly those visualising potential interactions between platelets and CTCs, need to be conducted to prove whether platelets can initiate or only maintain EMT in tumour cells during transit through the blood stream.

hPC is correlated with elevated levels of certain serum cytokines/chemokines, which support platelets' pro-tumorigenic function (ENA78/CXCL5, MMP1) and play a role in their activation (MDC/CCL22) [39]. Increased levels of ENA78/CXCL5 and IL17a are observed in patients with CTCs and hPC in contrast to those without CTCs or CTCs and nPC. Interestingly, some of these selected cytokines/chemokines (APRIL, HGF, IL17a and SCF) can stimulate megakaryocytopoiesis and/or regulate platelet production [40–43]. In the current study, both higher levels of IL17a and hPC correlated with a high content of intratumoural stroma within the primary tumour. If three patients with defined thrombocytosis are added to the group of patients with hPC, SCF correlated also with a higher stromal content (data not shown). Thus, it can be speculated that the stromal component of the primary tumour secretes mediators, which in turn can modulate the bone marrow function to guide tumour dissemination and prepare the bone marrow niche for subsequent nesting of tumour cells. Putatively stroma-derived IL17a or SCF can participate in such modulation (e.g. through increased production of platelets [42, 43] that later act as pro-tumorigenic in primary tumour and blood), which consequently leads to the formation of metastasis and patients' death (Fig. 3). In our study, as a proof-of-principle, a sub-analysis using the CIBERSORTx digital cytometry algorithm is performed to identify stromal component(s) potentially associated with hPC. The analysis results show that transcriptome signatures indicating the presence of resting mast cells and CD8+ T cells are correlated with both hPC and some identified platelet-associated cytokines. Both sub-populations of putatively identified cells can be strong activators of inflammation and produce IL17a [44–46]. Nevertheless, further in vivo studies and clinical sample analyses are required to gain sufficient knowledge on this phenomenon.

In conclusion, these outcomes indicate that the evaluation of platelets, both individually and in combination with CTCs, has prognostic potential in detecting non-metastatic breast carcinoma, particularly its luminal subtype.

The results also indicate the plausible link among the higher content of intratumoural stroma, hPC, CTCs and patients' worse outcomes. A detailed examination of individual blood components, such as blood cells and cytokines/chemokines, is required to reveal the complex network of environmental elements hypothetically supporting tumour progression. Further studies on larger cohorts of patients with different molecular subtypes of breast cancer are required to (i) specify the interactions between primary tumour and bone marrow modulated by secreted signalling molecules, such as cytokines/chemokines, and (ii) reveal the clinical relevance of anti-platelet therapies to prevent progression in breast cancer patients who have tested positive for hPC and/or CTCs.

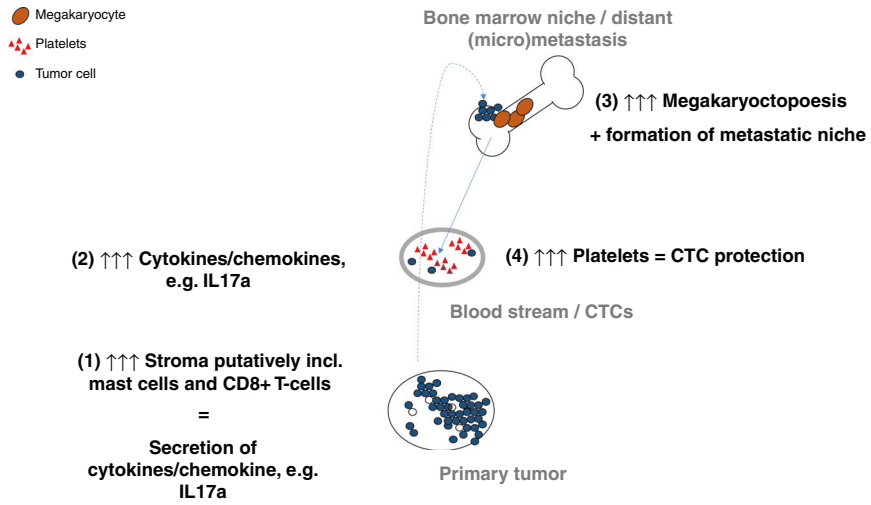


Fig. 3 Putative model of tumour progression involving regulation of platelet level by intratumoural stroma-derived factors. The stromal components of primary tumours (e.g. CD8 + T cells and/or mast cells) secrete mediators (e.g. specific cytokines/chemokines) (**1**), which in turn modulate the bone marrow function (**2**) to guide tumour dissemination and prepare the bone marrow niche for subsequent nesting of tumour cells (**3**). SCF, IL17a and APRIL can participate in such modulation through the stimulation of megakaryocytes, resulting in the production of platelets that later act as premetastatic (**4**).

DATA AVAILABILITY

The data that support the findings of this work are available from the corresponding author upon request. Immune-related transcriptome data are available at NCBI GEO under accession number GSE180186.

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AUTHOR CONTRIBUTIONS

Conceptualisation, NB-K and AJZ; methodology, NB-K, TK, JSz, DS and JL; formal analysis, NB-K and MP; investigation, NB-K, TK, JSz, MP, AM, DS and JL; resources, BK, KP, LK, JS and AJZ; data curation, NB-K and MP; writing — original draft preparation, NB-K and AJZ.; writing — review and editing, all authors; visualisation, NB-K and MP; supervision, NB-K and AJZ; project administration, AJZ; funding acquisition, AJZ.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Primary tumour samples and blood samples were collected from the patients according to a standard protocol approved by the Medical University of Gdansk ethics committee and upon signing an informed consent. This study was performed in accordance with the tenets of the Declaration of Helsinki.

CONSENT FOR PUBLICATION

All informed consent was obtained. This report does not contain any individual person's information.

ADDITIONAL INFORMATION

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Materiały uzupełniające do Publikacji 2

Supplementary Material

Higher platelets counts correlate to progression and can be induced by intratumoural stroma in non-metastatic breast carcinomas

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This file contains:

Supplementary Figure 3

Supplementary Figure 4

Supplementary Figure 5

Supplementary Table 1 Clinical data of breast cancer patients from Cohort I.

Due to the missing values not all numbers sum up to 70 cases.

		n	%
Age	<50	16	22.9
	>=50	54	77.1
T status	T1	31	44.9
	T2	33	47.8
	T3	3	4.3
	T4	2	2.9
N status	N0	35	50.0
	N1	35	50.0
Tumor grade	G1	11	15.7
	G2	32	45.7
	G3	27	38.6
Hormone receptor status	negative	14	20.0
	positive	56	80.0
Her2	negative	54	77.1
	positive	16	22.9
Stage 7th Edition AJCC	1a	21	30.0
	2a	21	30.0
	2b	13	18.8
	3a	8	11.6
	3b	2	2.9
	3c	4	5.8
Molecular subtype	luminal A	19	30.2
	luminal B/Her-	19	30.2
	luminal B/Her2+	11	17.5
	Her2+	5	7.9
	triple negative	9	14.3
Primary tumor phenotype	epithelial	41	58.6
	mesenchymal	29	41.4
CTC	no CTC	51	72.9
	epithelial CTC	9	12.9
	mesenchymal CTC	10	14.3
Stroma	low	53	75.7
	high	17	24.3
Death	no	61	87.1
	yes	9	12.9

Supplementary Table 2 Blood counts distribution in breast cancer patients from Cohort I.

Values written in italic indicate the outliers according to normal range described for women. Hb – hemoglobin, min – minimum, max – maximum.

	units	normal range (woman)	nr of patients with value <min normal range	min value	max value	nr of patients with value >max normal range	mean value	SD
erythrocytes	T/L	**3.50-5.20**	0	3.71	*5.49*	3	4.53	0.36
Hb	g/dL	**11-15.6**	3	*10.1*	*16.9*	5	13.63	1.24
hematocrit	%	**30-49.5**	0	31.3	48.7	0	40.63	3.36
leukocytes	G/L	**4.5-11**	2	*2.68*	*13.97*	1	6.99	1.80
monocytes	G/L	**0.2-0.8**	0	0.27	*0.91*	3	0.53	0.15
lymphocytes	G/L	**0.9-5**	1	*0.79*	4.23	0	2.07	0.78
neutrophils	G/L	**1.9-8**	2	*1.25*	*9.39*	1	4.23	1.40
eosinophils	G/L	**0.10-0.5**	0	0.00	*0.76*	2	0.13	0.13
basophils	G/L	**0-0.1**	0	0.00	0.06	0	0.02	0.01
platelets	G/L	**150-400**	0	160.00	190.00	0	257.72	51.26

Supplementary Table 3 Clinical data of breast cancer patients from Cohort II.

Due to the missing values not all numbers sum up to 254 cases.

		n	%
Age	<50	158	62.2
	>=50	96	37.8
T status	T1	127	50.2
	T2	95	37.5
	T3	24	9.5
	T4	7	2.8
N status	N0	123	49.6
	N1	125	50.4
Tumor grade	G1	37	15.2
	G2	113	46.5
	G3	93	38.3
Hormone receptor status	negative	89	36.0
	positive	158	64.0
Her2	negative	197	79.8
	positive	50	20.2
Molecular subtype	luminal A	128	51.8
	luminal B/Her-	19	7.7
	luminal B/Her2+	11	4.5
	Her2	39	15.8
	triple negative	50	20.2
Death	no	221	87.4
	yes	32	12.6
Platelets	nPC	204	80.0
	hPC	51	20.0

Supplementary Table 4 Comparison of platelets counts to clinical data of breast cancer patients from Cohort I.

hPC indicates high normal platelets count, nPC – normal platelets count, CTC – circulating tumor cells, neg – negative, pos – positive, F – Fisher exact test (if not mentioned – Chi-square test). Not all numbers sum up to 70 cases due to the missing values.

		nPC		hPC	
		n	%	n	%
Age	<50	13	23.6	3	20.0
	>=50	42	76.4	12	80.0
		p=1.00 (F)			
T status	T1	26	48.1	5	33.3
	T2	27	50.0	6	40.0
	T3	0	0	3	20.0
	T4	1	1.9	1	6.7
		p=0.006			
N status	N0	28	50.9	7	46.7
	N1	27	49.1	8	53.3
		p=0.771			
Tumor grade	G1	6	10.9	5	33.3
	G2	29	52.7	3	20.0
	G3	20	36.4	7	46.7
		p=0.032			
Hormone receptor status	negative	11	20.0	3	20.0
	positive	44	80.0	12	80.0
		p=1.000 (F)			
Her2 status	negative	42	76.4	12	80.0
	positive	13	23.6	3	20.0
		p=1.000 (F)			
Stage 7th Edition AJCC	1a	17	31.5	4	26.7
	2a	18	33.3	3	20.0
	2b	11	20.4	2	13.3
	3a	6	11.1	2	13.3
	3b	1	1.9	1	6.7
	3c	1	1.9	3	20.0
		p=0.120			
Molecular subtype	luminal A	12	24.5	7	50.0
	luminal B/Her-	18	36.7	1	7.1
	luminal B/Her2+	8	16.3	3	21.4
	Her2+	4	8.2	1	7.1
	triple negative	7	14.3	2	14.3
		p=0.224			
Primary tumor phenotype	epithelial	33	60.0	8	53.3
	mesenchymal	22	40.0	7	46.7
		p=0.642			
CTC	no CTC	43	78.2	8	53.3
	epithelial CTC	8	14.5	1	6.7
	mesenchymal CTC	4	7.3	6	40.0
		p=0.006			
Stroma	low	42	76.4	11	73.3
	high	13	23.6	4	26.7
		p=1.000 (F)			

Supplementary Table 5 Impact of PC/CTC status on OS of breast cancer patients in multivariate analysis.

Statistically significant results are presented in bold. HR – hazard ratio, CI – confidence interval, hPC – high normal platelet count, CTC – circulating tumour cells, neg – negative, pos – positive. Platelet range: 150,000-400,000, pts n = 70.

Parameter	Group	univariate			multivariate		
		p-value	HR	CI 95%	p-value	HR	CI 95%
Age	>=50 vs. <50	0.714	1.278	0.343-4.764	-	-	-
T status	**T3-4 vs. T1-2**	**<0.001**	**1.123**	**1.057-1.193**	0.073	1.084	0.992-1.185
N status	N1 vs. N0	0.099	3.751	0.778-18.085	-	-	-
Tumor grade	G3 vs. G1-2	0.723	0.974	0.842-1.127	-	-	-
Her 2 status	pos vs. neg	0.720	0.750	0.156-3.614	-	-	-
Platelets/CTC status	**hPC/CTC vs. others**	**0.001**	**9.452**	**2.532-35.282**	0.262	3.031	0.437-21.009

Supplementary Table 6 Comparison of platelets counts to clinical data of breast cancer patients from Cohort II.

hPC indicates high normal platelets count, nPC – normal platelets count, neg – negative, pos – positive, F – Fisher exact test (if not mentioned – Chi-square test). Not all numbers sum up to 254 cases due to the missing values.

		nPC		hPC	
		n	%	n	%
Age	<50	128	62.7	30	60.0
	>=50	76	37.3	20	40.0
		p=0.720			
T status	T1	100	49.3	27	54.0
	T2	80	39.4	15	30.0
	T3	17	8.4	7	14.0
	T4	6	3.0	1	2.0
		p=0.458			
N status	N0	96	48.5	27	54.0
	N1-2	102	51.5	23	46.0
		p=0.486			
Tumor grade	G1	25	13.0	12	24.5
	G2	94	48.7	19	38.8
	G3	74	38.3	18	36.7
		p=0.119			
Hormone receptor status	negative	70	35.4	19	38.8
	positive	128	64.6	30	61.2
		p=0.655			
Her2	negative	159	80.3	38	77.6
	positive	39	19.7	11	22.4
		p=0.668			
Molecular subtype	luminal A	101	51.0	27	55.1
	luminal B/Her-	19	9.6	0	0
	luminal B/Her2+	8	4.0	3	6.1
	Her2	31	15.7	8	16.3
	triple negative	39	19.7	11	22.4
		p=0.251			

Supplementary Table 7 Impact of platelets on OS of luminal breast cancer patients from Cohort II in multivariate analysis.

Statistically significant results are presented in bold. HR – hazard ratio, CI – confidence interval, hPC – high normal platelet count, nPC – normal platelet count, neg – negative, pos – positive. Platelet range: 150,000-400,000, pts n = 151.

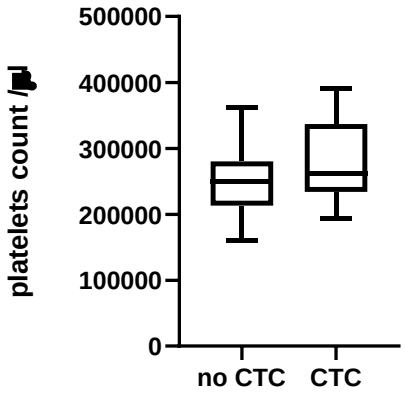
Parameter	Group	univariate			multivariate		
		p-value	HR	CI 95%	p-value	HR	CI 95%
Age	>=50 vs. <50	0.176	2.164	0.707-6.622	-	-	-
T status	**T3-4 vs. T1-2**	**<0.001**	**1.142**	**1.084-1.202**	**0.001**	**1.129**	**1.053-1.209**
N status	**N1 vs. N0**	**0.040**	**3.871**	**1.064-14.084**	0.666	1.507	0.234-9.712
Tumor grade	**G3 vs. G1-2**	**0.085**	**0.895**	**0.789-1.015**	-	-	-
Her 2 status	pos vs. neg	0.363	2.014	0.446-9.094	-	-	-
Platelets status	**hPC vs. nPC**	**0.067**	**2.841**	**0.929-8.693**	0.335	1.854	0.528-6.505

Supplementary Table 8 Impact of platelets on OS of breast cancer patients from Cohort II at age of >=50 in multivariate analysis.

Statistically significant results are presented in bold. HR – hazard ratio, CI – confidence interval, hPC – high normal platelet count, nPC – normal platelet count, neg – negative, pos – positive. Platelet range: 150,000-400,000, pts n = 92.

Parameter	Group	univariate			multivariate		
		p-value	HR	CI 95%	p-value	HR	CI 95%
T status	**T3-4 vs. T1-2**	**<0.001**	**1.129**	**1.065-1.197**	**0.001**	**1.115**	**1.046-1.189**
N status	N1 vs. N0	0.222	2.239	0.599-9.057	-	-	-
Tumor grade	G3 vs. G1-2	0.387	0.937	0.810-1.085	-	-	-
Hormone receptor status	neg vs. pos	0.402	26.592	0.012-57566	-	-	-
Her 2 status	pos vs. neg	0.339	0.36	0.045-2.913	-	-	-
Platelets status	**hPC vs. nPC**	**0.016**	**4.649**	**1.336-16.183**	0.331	1.973	0.501-7.771

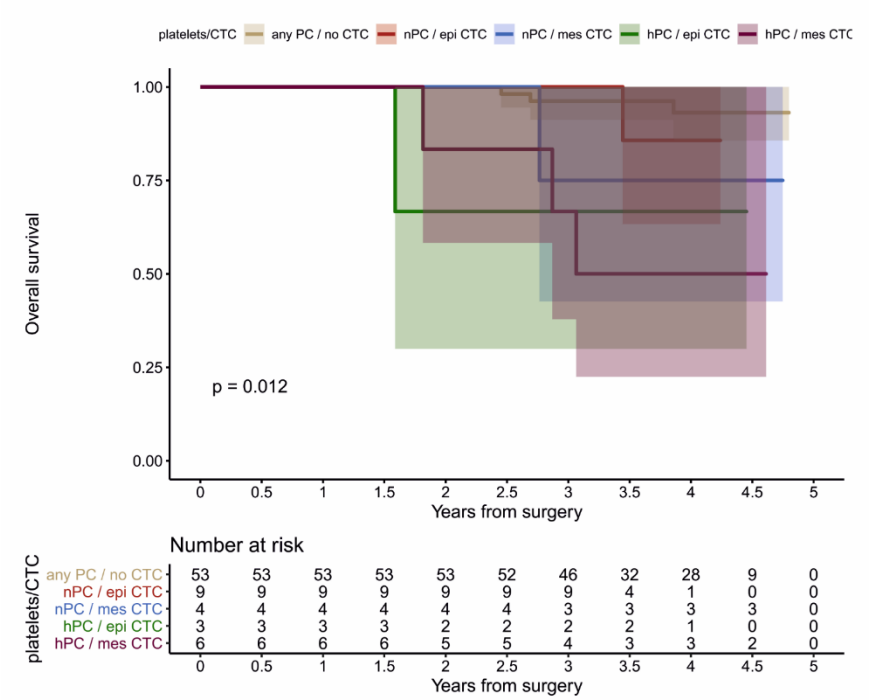
Supplementary Figure 1 Relationship between platelets and presence of CTC (box-plot, Mann-Whitney test, p= 0.125)



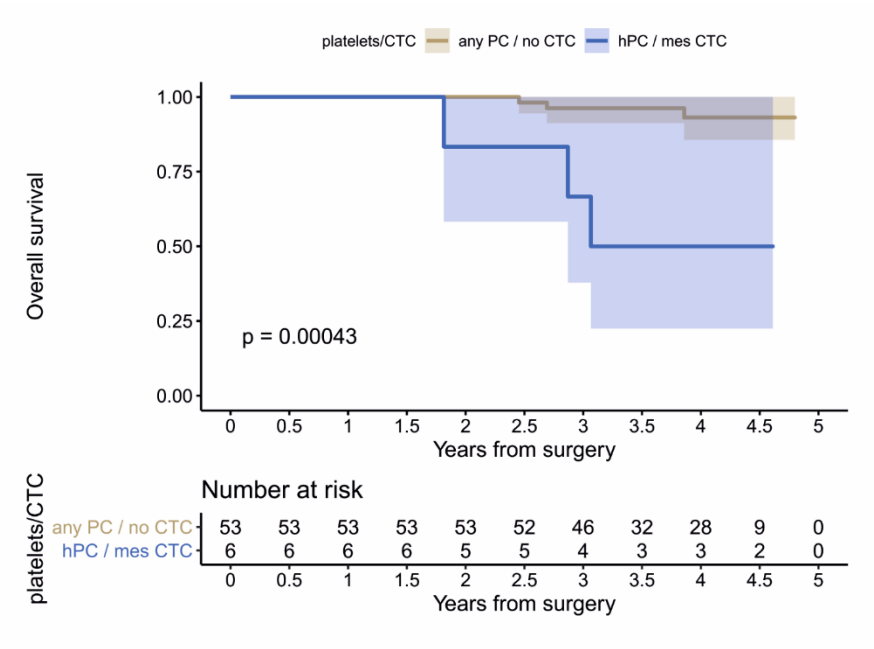
Supplementary Figure 2 Survival analysis of breast cancer patients (n = 70) in the contexts of combined platelet counts and CTC phenotype

hPC – high normal platelet count, nPC – normal platelet count, epi CTC – circulating tumour cell of epithelial phenotype, mes CTC – circulating tumour cell of mesenchymal phenotype, pts n = 70.

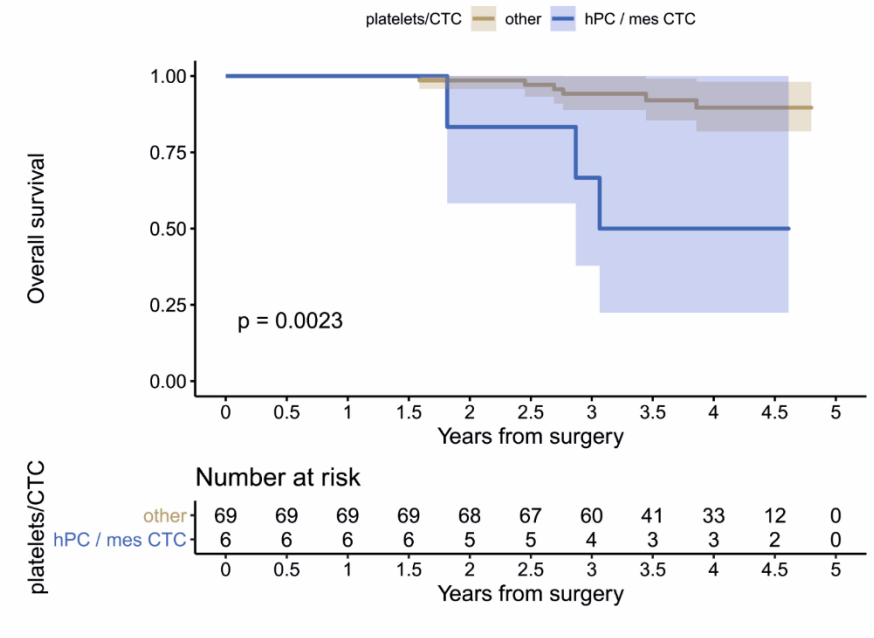
A



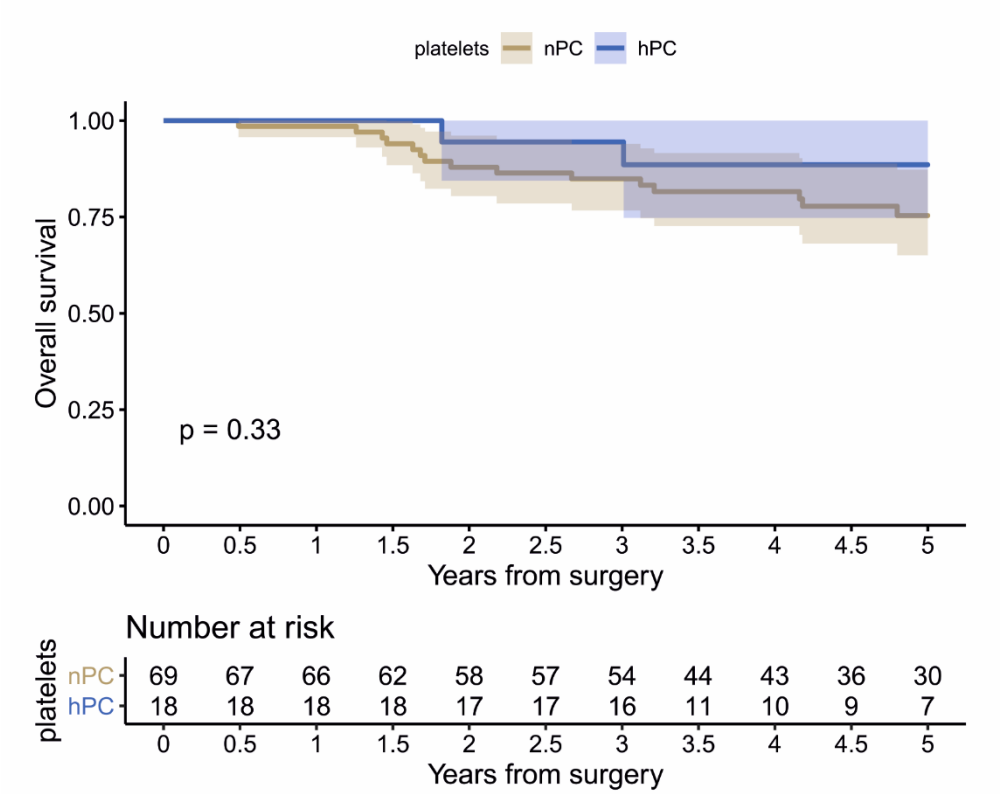
B


platelets/CTC
any PC / no CTC
hPC / mes CTC
Overall survival
1.00
0.75
0.50
0.25
0.00
p = 0.00043
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Years from surgery
Number at risk
platelets/CTC
any PC / no CTC
hPC / mes CTC
53 53 53 53 53 52 46 32 28 9 0
6 6 6 6 5 5 4 3 3 2 0
Years from surgery

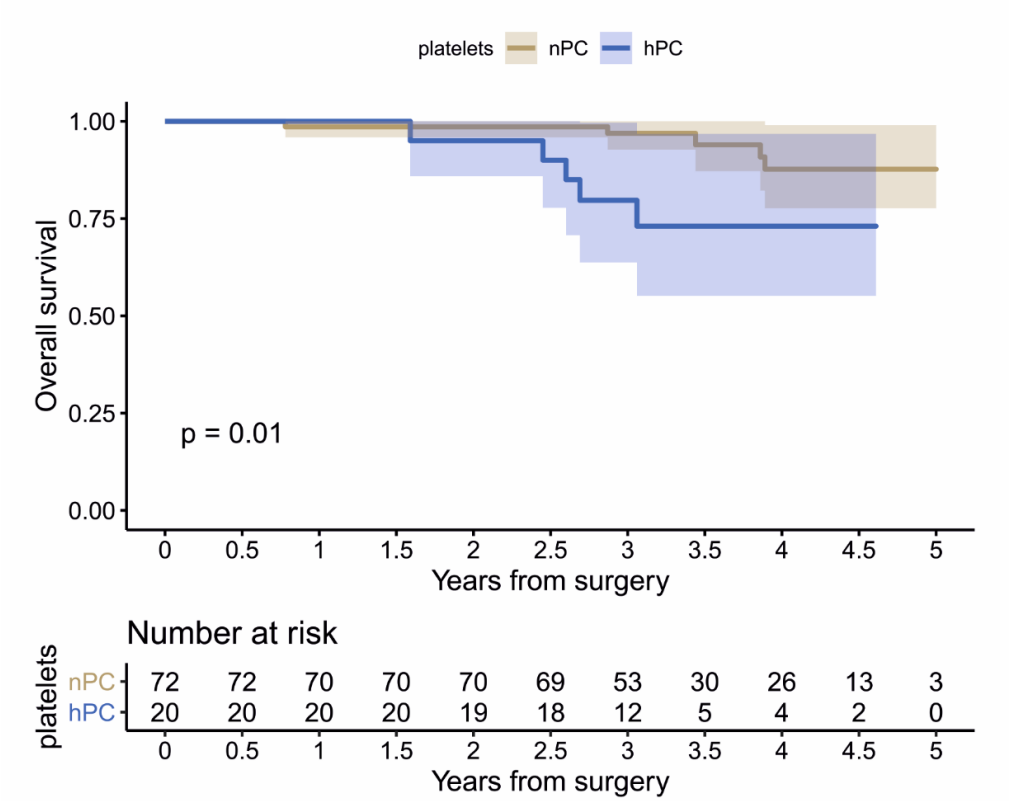
C


platelets/CTC
other
hPC / mes CTC
Overall survival
1.00
0.75
0.50
0.25
0.00
p = 0.0023
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Years from surgery
Number at risk
platelets/CTC
other
hPC / mes CTC
69 69 69 69 68 67 60 41 33 12 0
6 6 6 6 5 5 4 3 3 2 0
Years from surgery

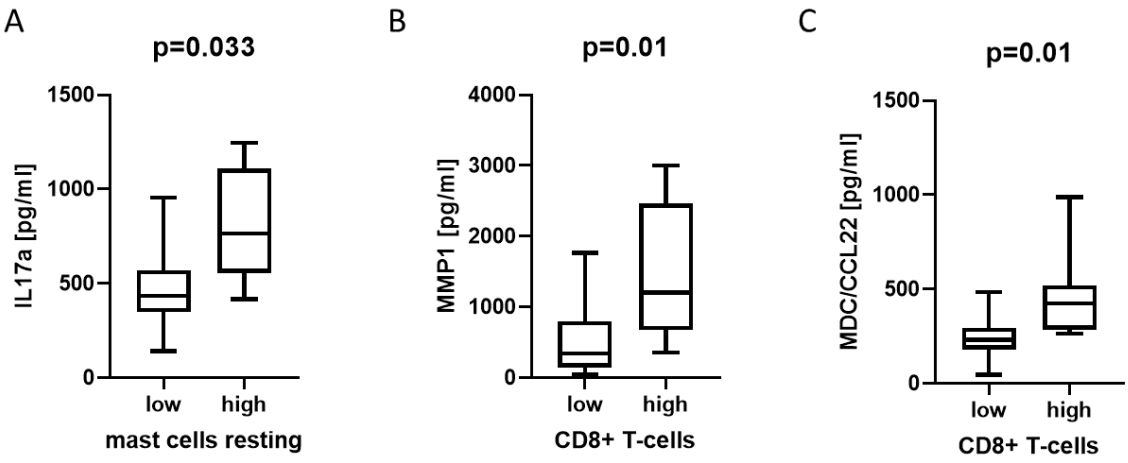
Supplementary Figure 3 Survival analysis of platelet count in patients with Her2+ or TNBC breast cancer (Cohort II, n=87)



Supplementary Figure 4 Survival analysis of platelet count in patients with breast cancer at age >=50 (Cohort II, n=92)



Supplementary Figure 5 Comparison of selected serum cytokines levels to resting mast cells and CD8+ T-cells signatures assessed within primary tumour.



Oświadczenia współautorów do Publikacji 2

Gdańsk, dnia 13.06.2022

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P2]

Bednarz-Knoll N, Popęda M, Kryczka T, Kozakiewicz B, Pogoda K, Szade J, Markiewicz A, Strzemecki D, Kalinowski L, Skokowski J, Liu J, Żaczek AJ

Higher platelet counts correlate to tumour progression and can be induced by intratumoural stroma in non-metastatic breast carcinomas

Br J Cancer. 2022 Feb;126(3):464-471. doi: 10.1038/s41416-021-01647-9

mój wkład obejmował:

- wykonanie analizy immunotranskryptomu (analiza bioinformatyczna, ustalenie składu populacyjnego nacieku infiltrującego guz); analizę statystyczną, opracowanie oraz interpretację tych wyników;
- zarządzanie danymi transkryptomicznymi;
- udział w przygotowaniu rycin i tabel;
- udział w odpowiedzi na recenzję.

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Oświadczam, iż w pracy [P2]

Bednarz-Knoll N, Popęda M, Kryczka T, Kozakiewicz B, Pogoda K, Szade J, Markiewicz A, Strzemecki D, Kalinowski L, Skokowski J, Liu J, Żaczek AJ

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mój wkład obejmował:

- opracowanie koncepcji badań;
- opracowanie metodologii analizy danych klinicznych, udział w opracowaniu metodologii analizy cytokin;
- wykonanie analizy danych klinicznych (w tym analiza przeżycia), analizę statystyczną, opracowanie oraz interpretację tych wyników; analizę statystyczną, opracowanie i interpretację wyników analizy cytokin i analizy histopatologicznej; udział w interpretacji wyników analizy immunotranskryptomu;
- zarządzanie danymi klinicznymi, histopatologicznymi oraz wynikami analizy cytokin;
- przygotowanie manuskryptu;
- przygotowanie rycin i tabel;
- przygotowanie odpowiedzi na recenzję;
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Bednarz-Knoll N, Popęda M, Kryczka T, Kozakiewicz B, Pogoda K, Szade J, Markiewicz A, Strzemecki D, Kalinowski L, Skokowski J, Liu J, Żaczek AJ

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mój wkład obejmował:

- opracowanie metodologii analizy histopatologicznej (oznaczenie zawartości podścieliska);
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mój wkład obejmował:

- wykonanie izolacji i analizy CTC; opracowanie oraz interpretację tych wyników.

Aleksandra Markiewicz

(podpis)

Gdańsk, dnia 13.06.2022

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P2]

Bednarz-Knoll N, Popęda M, Kryczka T, Kozakiewicz B, Pogoda K, Szade J, Markiewicz A, Strzemecki D, Kalinowski L, Skokowski J, Liu J, Żaczek AJ

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mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- redakcję manuskryptu;
- udział w odpowiedzi na recenzję;
- nadzór merytoryczny nad przygotowaniem publikacji;
- pozyskanie środków na badania i kierowanie projektem;
- zapewnienie zaplecza badawczego, w tym niezbędnej infrastruktury;
- koordynację działań projektowych, w tym pozyskania materiału klinicznego, danych klinicznych i histopatologicznych oraz analizy molekularnej.

(podpis)

Publikacja 3 [P3]

Markiewicz A, Topa J, **Popęda M**, Szade J, Skokowski J, Wełnicka-Jaśkiewicz M, Żaczek A

Activation of epithelial-mesenchymal transition process during breast cancer progression – the impact of molecular subtype and stromal composition

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Regular paper

Activation of epithelial-mesenchymal transition process during breast cancer progression – the impact of molecular subtype and stromal composition*

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Breast cancer (BC) is a heterogeneous disease with different molecular subtypes, which can be defined by oestrogen (ER), progesterone (PR) and human epidermal growth factor (HER2) receptors' status as luminal, HER2+ and triple negative (TNBC). Molecular subtypes also differ in their epithelial-mesenchymal phenotype, which might be related to their aggressiveness, as activation of the epithelial-mesenchymal transition (EMT) is linked with increased ability of cancer cells to survive and metastasize. Nevertheless, the reverse process of mesenchymal-epithelial transition was shown to be required to sustain metastatic colonization. In this study we aimed to analyse activation of the EMT process in primary tumours (PT), which have (N+) or have not (N–) colonized the lymph nodes, as well as the lymph nodes metastases (LNM) themselves in 88 BC patients. We showed that luminal N– PT have the lowest activation of the EMT process (27%), in comparison to N+ PT (48%, *p*=0.06). On the other hand, TNBC do not show statistically significant EMT activation at the stage before lymph colonization (N–, 83%) and after colonization of the lymph nodes (N+, 63%, *p*=0.58). TNBC are also the least plastic (unable to change the EMT phenotype) in terms of turning EMT on or off between matched PT and LNM (0% EMT plasticity in TNBC *vs* 36% plasticity in luminal tumours). Moreover, in TNBC activation of EMT was correlated with increased cell division rate of the PT– in mesenchymal TNBC PT median Ki-67 was 45% in comparison to 10% in epithelial TNBC PT (*p*=0.002), whereas in PT of luminal subtypes Ki-67 did not differ between epithelial and mesenchymal phenotypes. Profiling of immunotranscriptome of epithelial and mesenchymal luminal BC with Nanostring technology revealed that N– PT with epithelial phenotype were enriched in inflammatory response signatures, whereas N+ mesenchymal cancers showed elevated MHC class II antigen presentation. Overall, activation of EMT changes during cancer progression and metastatic colonization of the lymph nodes depending on the PT molecular subtype and is related to differences in stromal signatures. Activation of EMT is associated with colonizing phenotype in luminal PT and proliferative phenotype of TNBC.

Key words: breast cancer, epithelial-mesenchymal transition, molecular subtypes, metastasis

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***This paper is dedicated to Professor Wacław Tadeusz Szybalski on the 100th anniversary of his birth**

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Abbreviations: BC, breast cancer; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer; EMT, epithelial-mesenchymal transition; PT, primary tumour; LNM, lymph node metastases; MET, mesenchymal-epithelial transition; TF, transcription factors; TWIST1, Twist-related protein 1; SNAI1 (SNAIL), Zinc finger protein SNAI1; SNAI2 (SLUG), Zinc finger protein SNAI2; ZEB1, Zinc finger E-box-binding homeobox 1; ZEB2, Zinc finger E-box-binding homeobox 2; OVOL1/2, ovo like transcriptional repressors 1/2; GRHL2, grainyhead like transcription factor 2; FFPE, formalin fixed and paraffin embedded; IHC, immunohistochemistry; VIM, vimentin; TMA, tissue microarrays; EPI, epithelial; MES, mesenchymal; DEG, differentially expressed gene; OS, overall survival; IFNγ, interferon gamma; TNFα, tumour necrosis factor alpha; IL-6, interleukin 6; TGFβ, transforming growth factor beta; IFITM2, Interferon Induced Transmembrane Protein 2; TRIF, TIR-domain-containing adapter-inducing Interferon-β; IFNβ, interferon beta; PAI-1, Plasminogen Activator Inhibitor-1

INTRODUCTION

Breast cancer (BC) is a heterogeneous disease with five different molecular subtypes – luminal (further subdivided to luminal A, luminal B HER2+, luminal B HER2–), HER2+, and basal subtypes/triple negative (TNBC) (Sarrió *et al.*, 2008; Cancer Genome Atlas Network, 2012; Kast *et al.*, 2015). These subtypes are the basis for prognostication and therapy selection. Of all the subtypes, luminal tumours, characterised by presence of hormone receptors, have the best prognosis (though they are also the ones which show late recurrence), whereas TNBC are more aggressive, with limited access to targeted treatment options (Hennigs *et al.*, 2016). Apart from having different profiles of growth hormone receptors, molecular subtypes differ in their invasiveness, stem cell phenotype and therapy resistance, which was attributed to the activation of the epithelial-mesenchymal transition (EMT) (Mani *et al.*, 2008; Morel *et al.*, 2008; Felipe Lima *et al.*, 2016; Shibue & Weinberg, 2017; Katsuno *et al.*, 2019). EMT is an early morphogenic program also activated under (patho)physiological conditions in adult tissues, which allows polarized and immobile epithelial cells to acquire features of motile mesenchymal cells

(Thiery, 2002). Ability to invade surrounding tissues, a feature characteristic for invasive cancers, is increased in tumour cells with activated EMT program (Sánchez-Tilló *et al.*, 2011; Lamouille *et al.*, 2014). Undergoing EMT and the reverse process of mesenchymal-epithelial transition (MET) is regulated by the action of EMT transcription factors (TF) and miRNA. Some TF promote EMT (such as Twist-related protein 1 – TWIST1, Zinc finger proteins SNAI1 and SNAI2, also referred to as SNAIL and SLUG, Zinc finger E-box-binding homeobox 1 and 2 – ZEB1, ZEB2), whereas others inhibit it (eg. ovo-like transcriptional repressors 1/2, OVOL1/2, grainyhead like transcription factor 2, GRHL2) (Roca *et al.*, 2013; Somarelli *et al.*, 2016). Similarly, miRNA can inhibit EMT (miR-205 and miR-200 family) or promote it (miR-9 and miR-155) (Burk *et al.*, 2008; Gregory *et al.*, 2008; Kong *et al.*, 2008; Gregory *et al.*, 2011; Zhang & Ma, 2012).

Activation of EMT can also be studied in cancers by the analysis of EMT effectors (which are regulated by EMT TF/miRNAs) – downregulation of epithelial markers (e.g. E-cadherin, claudins, occludins) and upregulation of mesenchymal markers (e.g. vimentin, N-cadherin, fibronectin) (Jechlinger *et al.*, 2003; Mani *et al.*, 2008; Moreno-Bueno *et al.*, 2008). It was also recognised that activation of EMT in cancer cells upregulates stem cell-like features and leads to therapy resistance (Mani *et al.*, 2008). Despite contribution of EMT to the metastatic dissemination, EMT process needs to be reversed *via* MET in order to allow metastatic colonization at a distant site (Gao *et al.*, 2012; Ocaña *et al.*, 2012). This means that EMT activation is crucial for dissemination, but MET is required for re-establishing epithelial phenotype and colonization of a new niche (Aiello & Kang, 2019). Therefore, transition between EMT and MET should provide plasticity necessary for dissemination from the primary tumour and colonization of a distant site. To test how EMT status of BC changes during metastatic progression, we have analysed EMT activation in non-colonizing PT (N–), colonizing PT (N+) and matched LNM, all in the context of two BC molecular subtypes (luminal and TNBC), which are known to differ in their EMT status.

MATERIALS AND METHODS

Patients and tested samples

Primary tumours (PT) of luminal and triple negative molecular subtypes and non-lobular histology (N=88), and matched lymph node metastases (LNM, N=41) from 88 non-metastatic BC patients were investigated. Patients were treated at the Medical University Hospital in Gdańsk between 2011 and 2013 according to the current standard of care. PT and LNM were removed during surgery and evaluated by a pathologist, followed by formalin fixation and paraffin embedding (FFPE), as described before (Markiewicz *et al.*, 2014). Staging was performed according to the classification of American Joint Committee on Cancer version 7 staging manual, and tumour grade was assessed according to the modified Bloom-Richardson system. Molecular subtype was assessed according to St Gallen criteria (Goldhirsch *et al.*, 2011) using oestrogen (ER) and progesterone (PR) receptors' status analysed by IHC and Allred scoring system; human epidermal growth factor receptor (HER2) status was analysed by immunohistochemistry (IHC) and fluorescent *in situ* hybridization in inconclusive cases (2+ IHC staining) and Ki-67. The ER, PR, and HER2 status were analysed during routine pathological examination of the samples, Ki-67 was tested by IHC on tissue microarrays (clone MIB-1, Dako, Copenhagen, Denmark), as described before (Markiewicz *et al.*, 2014). All luminal tumours (luminal A, luminal B) were combined into one group, further described as the luminal subtype. Median age of the patients was 61 years and median follow up time (overall survival) was 4.1 years. Fifty-one percent (45/88) of the patients had LNM (detailed clinico-pathological characteristics of patients are presented in Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). The study was accepted by the Independent Ethics Committee of the Medical University of Gdańsk.

Immunohistochemical analysis of PT and LNM

Whole FFPE sections of PT and LNM were subjected to IHC staining of E-cadherin (clone NCH 38, Dako), N-cadherin (clone 6G11, Dako) and Vimentin (VIM; clone V9, Dako), as described and presented in our previous work (Markiewicz *et al.*, 2014). Activation of EMT (mesenchymal status of a sample) was defined as either E-cadherin loss in at least 10% of the cancer cells or acquisition of N-cadherin or VIM in at least 10% of the cancer cells in the evaluated PT/LNM section. All three markers (E-cadherin, N-cadherin, VIM) had to be evaluated to assign EMT status of a sample, either epithelial (EPI) or mesenchymal (MES). Stroma content was assessed in tissue microarrays (TMA) comprised of five 1-mm diameter tumour samples per each patient (Markiewicz *et al.*, 2014) based on hematoxylin-eosin staining. For each specimen, the maximum record of stroma content out of all evaluated and informative tissue cores was assigned for further analysis.

Immune-related transcriptome profiling with nCounter technology

Transcriptome analysis was performed for N- PT (N=11, including 7 with epithelial and 4 with mesenchymal status), N+ PT (N=23, including 10 with epithelial and 13 with mesenchymal status) and LNM (N=11, including 7 with epithelial and 4 with mesenchymal status) fragments, as previously described (Popeda *et al.*, 2019). In brief, total RNA was extracted from FFPE blocks with RNeasy Mini Kit (Qiagen, Germantown, MD, USA), followed by preamplification and measurement of 730 immune-related genes' expression (nCounter PanCancer Immune Profiling Panel, NanoString Technologies, Seattle, WA, USA). Background correction and normalization were conducted with the nSolver 4.0 software (NanoString Technologies) according to the manufacturer's recommendations. Following low-expression gene filtering (global log2 mean count <6), 593 genes were included in the final analysis (Popeda *et al.*, 2021). The NanoString platform is highly comparable with golden standard gene expression approach – RT-qPCR, and it might even outperform it on low-quality material like FFPE samples (Reis *et al.*, 2011; Veldman-Jones *et al.*, 2015). Raw expression data were submitted to NCBI GEO database under GSE180186 accession number.

STATISTICAL ANALYSIS

Data were analysed using the R statistical environment (version 3.6.1), GraphPad online tool and STATISTICA software (version 13.0, Statsoft, Cracow, Poland). Results were visualized with GraphPad Prism (version 8, Graph-

Pad Software, Inc., San Diego, CA, USA) licensed for Medical University of Gdańsk.

Categorical variables were compared by Pearson's chi-squared and Fisher's exact test. Differences between quantitative values (gene expression levels between tissues of epithelial and mesenchymal phenotype) were estimated with Mann–Whitney U test, with p-values <0.05 considered as statistically significant. Differentially expressed gene (DEG) status was inferred based on statistical significance. Genes with median-based log2FC$\geq$1 were considered as up- and genes with log2FC$\leq$–1 as down-regulated. For each type of tissue, DEGs were associated with GO BP and Reactome terms using Functional Annotation Tool by DAVID Bioinformatics Resources 6.8 (Huang *et al.*, 2009a; Huang *et al.*, 2009b). Kaplan-Meier curves for overall survival (OS) were compared using a log-rank test. Cohen's kappa was used to measure agreement between the EMT status of PT and LNM (Landis & Koch, 1977).

RESULTS

EMT activation during metastatic progression

EMT activation was defined as either loss of E-cadherin or expression of VIM or N-cadherin (independently of the E-cadherin status) in the IHC staining of the LNM, as well as PT which have (N+) or have not colonized the lymph nodes (N–). With these criteria, EMT activation occurred in 35% N– PT and 51% N+ PT (p=0.13), as well as in 32% of LNM (Fig. 1A). LNM showed a decreased activation of EMT in comparison to matched PT (N+, p=0.07, Fig. 1A). When samples were analysed with subdivision into molecular subtypes, a large disproportion in the EMT activation status was noted between luminal and TNBC (27% and 83%, respectively; p=0.01, Fig. 1B) in the N– PT, but not in the N+ PT

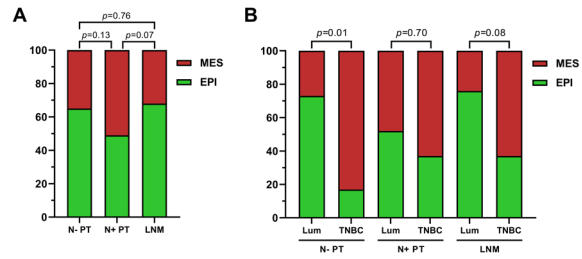

Figure 1. Activation of the EMT process in PT and LNM.
Percentages of epithelial (EPI) and mesenchymal (MES) phenotypes of PT which have not (N-) or have (N+) metastasized to the lymph nodes, as well as lymph node metastases themselves (LNM) presented without (**A**) and with subdivision to molecular subtypes (**B**). Significance levels were calculated with Pearson's chi-squared or Fisher's exact test.

(48% in luminal and 63% in TNBC; p=0.70, Fig. 1B). In LNM, similarly to N– PT, a trend towards disproportion in EMT activation between molecular subtypes occurred (24% in luminal and 63% in TNBC, p=0.08, Fig. 1B). In other words, the data show that during metastatic progression and lymph node colonization the EMT status of cancer cells changes, but to a different degree depending on the molecular subtype of the tumour. Luminal PT are more prone to turn on EMT during cancer progression, as shown by the increase in the mesenchymal status by 21% from N– to N+ stage (p=0.06, Fig. S1A at https://ojs.ptbioch.edu.pl/index.php/abp/). For TNBC, the change in the EMT status between N– and N+ was not significant (p=0.58, Fig. S1B at https://ojs.ptbioch.edu.pl/index.php/abp/). During lymphatic colonization, luminal cancers turn off EMT – LNM showed a decreased mesenchymal status in comparison to matched PT (N+) (24% in LNM *vs* 48% in PT, p=0.04, Fig. S1A at https://ojs.ptbioch.edu.pl/index.php/abp/), but no change in EMT status is observed in the TNBC subtype (mes-

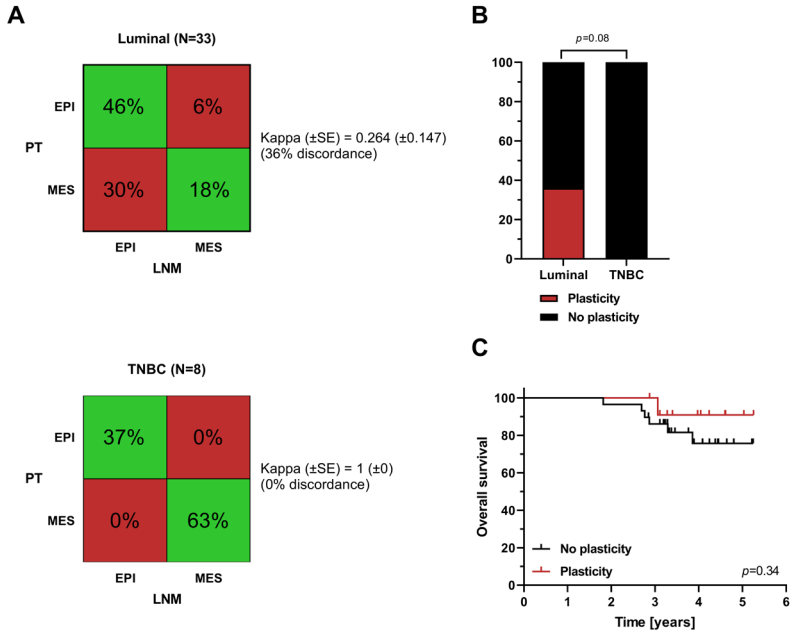

Figure 2. EMT plasticity in molecular subtypes of breast cancer.
Percentage of EMT phenotype changes between N+ PT and LNM of luminal and TNBC subtype (A; Cohen's kappa); occurrence of EMT phenotype change (EMT plasticity) between N+ PT and LNM in luminal and TNBC cancers (B; Fisher's exact test); prognostic significance of EMT plasticity in luminal and TNBC cancers – effect on overall survival of the patients (C; log-rank test).

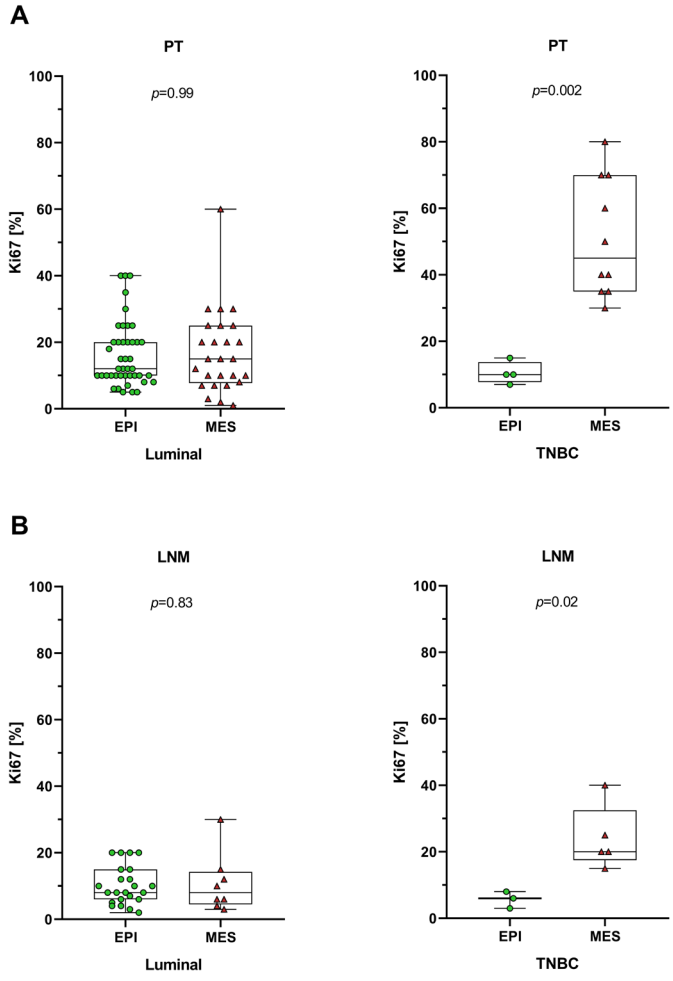

Figure 3. Cancer cell division rate in PT and LNM of luminal and TNBC subtypes.
Ki-67 staining in (**A**) primary tumours or (**B**) lymph node metastases. Significance levels were calculated with Mann-Whitney U test.

Table 1. Correlation between clinico-pathological data of patients and occurrence of EMT plasticity between N+PT and LNM.
P was calculated with Pearson's chi-squared or Fisher's exact test.

Variable	Status	EMT plasticity		% of samples with EMT plasticity	*p*
		No	Yes		
Age	≤50 years	10	4	29	1.00
	>50 years	19	8	30	
Tumour size (T)	T1-2	26	11	30	1.00
	T3	2	1	33	
Number of involved lymph nodes	≤3	19	4	17	0.06
	>3	10	8	44	
Grade	G1-2	13	9	41	0.08
	G3	16	3	16	
ER	Negative	9	1	10	0.23
	Positive	20	11	35	
PR	Negative	11	1	8	0.07
	Positive	18	11	38	
HER2	Negative	20	8	29	1.00
	Positive	9	4	31	

enchymal status in 63% in LNM *vs* 63% in PT, p=1.00, Fig. S1B at https://ojs.ptbioch.edu.pl/index.php/abp/).

Clinical significance of EMT plasticity during metastatic colonization

To evaluate more closely the change in the EMT status between N+ PT and LNM in different molecular subtypes, we evaluated the EMT plasticity which we defined as the occurrence of any EMT phenotypic switch between N+ PT and LNM compartment. In luminal cancers, 36% of the samples had discordant EMT activation status (Fig. 2A; usually a switch from mesenchymal status in PT to epithelial in LNM – 30% of the cases), whereas in the TNBC no PT-LNM discordance occurred (Fig. 2A). Calculated Cohen's kappa, which measures agreement between the EMT status of PT and LNM, showed perfect concordance in TNBC subtype (k=1), but fair agreement in luminal cancers (κ=0.264, Fig. 2A). This indicates greater EMT plasticity in luminal than in TNBC tumours during lymphatic spread (Fig. 2B).

EMT plasticity was associated with the presence of progesterone receptors (p=0.07), higher number of involved lymph nodes (p=0.06), and lower tumour grade (p=0.08), with 41% of the tumours showing EMT plasticity in the G1-G2 group, in comparison to 16% of the tumours with low differentiation (G3) (p=0.08; Table 1). EMT plastic tumours showed better overall survival than tumours which did not change EMT status between N+ PT and LNM, though the results did not reach statistical significance (p=0.34, Fig. 2C). At the same time, the EMT status of PT or LNM was not affecting the overall survival of the patients (Fig. S2A and S2B at https://ojs.ptbioch.edu.pl/index.php/abp/).

Interestingly, we observed that the EMT status of PT was related to differences in cell division rate depending on the molecular subtype of the tumour. In TNBC, mesenchymal phenotype of the PT resulted in 4.5-times higher cell division rate than in the epithelial phenotype (median Ki-67 – 45% in mesenchymal PT *vs* 10% in epithelial PT, p=0.002; Fig. 3A). Similar observation was made in the LNM (median Ki-67 – 20% in mesen-

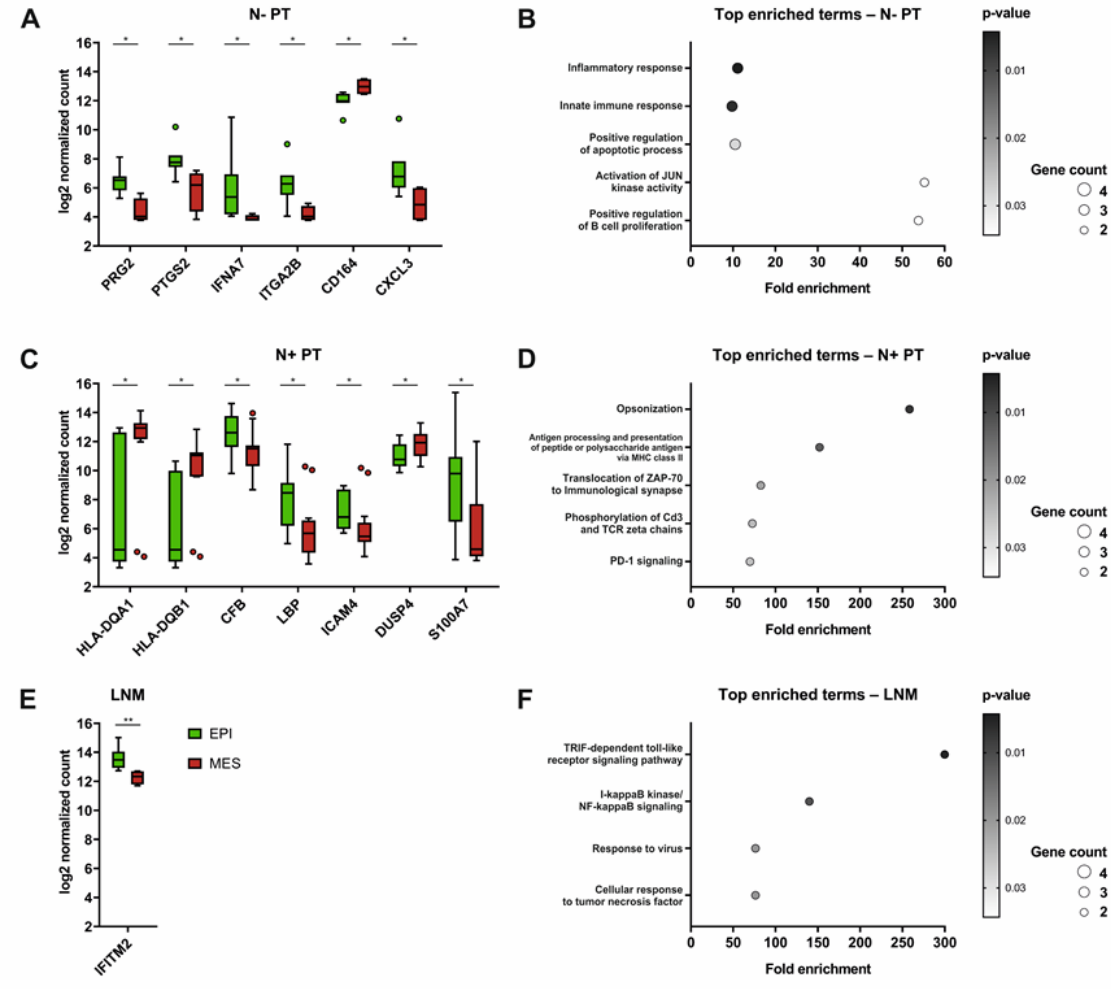

Figure 4. Differentially expressed immune-related genes between breast cancer samples of epithelial (EPI) and mesenchymal (MES) EMT phenotype assessed in PT N– (A), PT N+ (C) and LNM (E).
Only significantly up- (log2FC≥1) and down-regulated (log2FC≤–1) DEGs are presented. Differences in median normalized counts between groups were analysed with the Mann–Whitney U test; *p<0.05, **p<0.01; the bars correspond to the interquartile range (IQR), the whiskers cover 1.5 IQR from the median. Top-enriched GO BP and Reactome terms among DEGs in each group of tissues – PT N– (B), PT N+ (D) and LNM (F). These were established using the Functional Annotation Tool by DAVID Bioinformatics Resources 6.81. Terms are plotted against fold enrichment and arranged in ascending order by p-value; dot size represents the number of genes associated with a given term, while dot colour represents the p-value.

chymal LNM *vs* 6% in epithelial LNM, p=0.02, Fig. 3B). No such differences were observed in PT or LNM of the luminal subtype (Fig. 3A and B).

Tumour stroma profiling in PT and LNM in the context of EMT activation

EMT can be induced by tumour microenvironment, and also by immune cells which secrete potent EMT inducers, like interferon gamma (IFNγ) (Cohen *et al.*, 2015), tumour necrosis factor alpha (TNFα) (Cohen *et al.*, 2015), interleukin 6 (IL-6) (Cohen *et al.*, 2015), and transforming growth factor beta (TGFβ) (Pang *et al.*, 2016; Kariche *et al.*, 2019). Therefore, we have asked if there are differences in the immunotranscriptome of PT and LNM, which show EMT activation *vs* those with no signs of EMT. The analysis was performed separately for N- and N+ PTs, as there were differences in EMT activation in these two groups as assessed by IHC (Fig. 1B), and also for N+ PT and LNM, which are an exact matched set originating from the same patients, therefore reflecting spatial and temporal changes of the tumour.

In the case of the N– PT, 5 genes were significantly down-regulated (log2FC≤–1) in mesenchymal, when compared to epithelial tumours, with just one gene (*CD164*) showing a significantly higher expression (log2FC≥1) in mesenchymal N– PT (Fig. 4A). In terms of Gene Ontology (GO), a substantial change in inflammatory response was observed (Fig. 4B), however, the genes identified as differentially expressed between epithelial and mesenchymal N– PT showed an ambiguous role in inflammation, possibly reflecting heterogeneous composition of the tumour stroma. Sialomucin *CD164*, the only gene significantly upregulated in N– PT of the mesenchymal phenotype, was shown to drive the mesenchymal-epithelial transition when expressed in lung cancer cells (Chen *et al.*, 2017), thus possibly playing a role in reverting aggressive mesenchymal phenotype of cancer cells to the epithelial phenotype.

In the N+ PT, the most significantly differentially expressed genes, with higher expression in mesenchymal PT, were MHC II molecules – *HLA-DQA1* (log2FC=8.38) and *HLA-DQB1* (log2FC=6.49, Fig. 4C), which was also reflected in the GO analysis, showing top enriched terms being related to antigen processing and presentation or T-cell associated signalling (Fig. 4D). At the same time, we have observed that mesenchymal PT from N+ patients had higher stroma content than epithelial tumours (Fig. S3 at https://ojs.ptbioch.edu.pl/index.php/abp/), which could indicate that the mesenchymal phenotype of PT is associated with higher infiltration of antigen presenting cells.

LNM showed only one significantly differentially expressed gene, which had higher expression in the epithelial than mesenchymal samples – *IFITM2* (Interferon Induced Transmembrane Protein 2, log2FC=–1.11, Fig. 4E). As *IFTM2* is an interferon-inducible gene, its increased expression suggests high interferon levels in epithelial LNM. Similarly, GO analysis showed TIR-domain-containing adapter-inducing IFNβ (TRIF)-dependent signalling pathway enrichment (Fig. 4F), which triggers production of type I interferon, especially interferon beta (IFNβ) (Yamamoto *et al.*, 2003).

Full list of differentially expressed genes and associated GO is shown in Table S2 and Table S3 at https://ojs.ptbioch.edu.pl/index.php/abp/, respectively.

DISCUSSION

Development of metastasis remains the biggest challenge in management of cancer (Klein, 2020). Therefore, mechanisms employed by BC to spread, avoid apoptosis/senescence and colonize are intensively studied. Activation of the EMT program was found to contribute by a number of mechanisms to the malignancy of cancer cells, hence recognizing its role in clinical samples is required to pinpoint features important for metastatic progression in cancer patients.

In this study we evaluated how activation of the EMT program changes during cancer progression and metastatic colonization of the lymph nodes in the context of BC molecular subtypes. We have found that during cancer progression (comparison of N– and N+ PT) the EMT status of PT changes; in luminal N– PT epithelial phenotype is clearly dominating, whereas in N– TNBC PT the mesenchymal phenotype is mostly observed. However, as PT progresses and seeds metastases to lymph nodes (N+ stage), the disproportion in the EMT status between luminal and TNBC molecular subtypes disappears. To our knowledge, this is the first report showing the difference in EMT activation status in BC molecular subtypes in the context of cancer progression. TNBCs are known to have more mesenchymal phenotype than luminal cancers (Blick *et al.*, 2008; Taube *et al.*, 2010; Tan *et al.*, 2014). However, our data show that these differences might be most prominent in PT that have not spread to the lymph nodes. In luminal BC, activation of the EMT process might be more important for metastatic spread than in TNBC. In line with this finding, Savci-Heijink and others have found by profiling PT (classified to molecular subtypes by PAM50) of metastatic BC patients (which more closely resemble N+ PT) that luminal PT had an increased level of EMT markers (84.6% of luminal A tumours, 65.1% of luminal B tumours, with the latter showing higher PR and cell cycle-related genes in comparison to luminal A tumours (Parker *et al.*, 2009) than basal PT (25%) (Savci-Heijink *et al.*, 2019).

We also evaluated plasticity (change) in the EMT status between matched PT-LNM pairs and found that it only occurred in luminal tumours and was connected with better differentiation of PT. This could indicate that well differentiated tumours have greater ability to switch between phenotypes than high grade tumours. Our previous study on similar group of patients showed that well differentiated tumours have higher expression of EMT core regulator, TWIST1 (Markiewicz *et al.*, 2014), which maintains EMT plastic state in breast cancer (Xu *et al.*, 2017). Nevertheless, the occurrence of EMT plasticity between PT and LNM was also connected with a higher number of involved lymph nodes, which might suggest that the ability to change EMT status (mostly switching EMT off in LNM) could support dissemination and metastatic colonization within the lymphatic system. Ocaña and others also found that the metastatic spread within the lymphatic system might not require EMT activation (Ocaña *et al.*, 2012), as the structure of the lymphatic compartment does not require intravasation of cells, which is normally enhanced by the EMT process. Therefore, EMT activation in the lymphatic system might not be required, but also might not be supported by the lymphatic environment. Indeed, our immunotranscriptome profiling of the LNM revealed that the epithelial phenotype of cancer cells might be forced by the TRIF-dependent signalling pathway, which is linked with the activation of type I interferon, like IFNβ. As IFNβ signalling pathway was shown to be decreased in

mammary cells with a mesenchymal phenotype (Doherty *et al.*, 2017) and repression of aggressive stem cell phenotype in BC (Doherty *et al.*, 2019), it would explain increased TRIF-dependent signalling in LNM with a less malignant epithelial phenotype.

Change in the EMT status of cancer cells might be a sign of cancer cells responsiveness to changes in the microenvironment, which can influence the EMT phenotype of the tumours (Quail & Joyce, 2013; Hussain *et al.*, 2020). During tumour progression changes in the microenvironment occur, which could exert different EMT-induction potential in cancer cells (Sica *et al.*, 2008; Hussain *et al.*, 2020; Westergaard *et al.*, 2020). By profiling immunotranscriptome of the BC samples we showed that there was no overlap in differentially expressed genes between epithelial and mesenchymal phenotypes in neither of the compartments (PT *vs* LNM) and stages of tumour progression (N– PT *vs* N+ PT). This might reiterate heterogeneity in EMT-inducing factors, which can be different in dynamically changing tumour microenvironment (Whiteside, 2008; Binnewies *et al.*, 2018). Two of the most significantly differentially expressed genes, increased in mesenchymal N+ PT, were *HLA-DQA1* and *HLA-DQB1*, being part of MHC class II antigen presentation complex. *In vitro* studies showed that macrophages, which are antigen presenting cells expressing MHC class II (Cruse *et al.*, 2004), can induce EMT in luminal BC cell lines (Bednarczyk *et al.*, 2018) and other cancers (Bonde *et al.*, 2012).

Though the reverse to the EMT process of mesenchymal-epithelial transition is believed to be required for effective proliferation (Gao *et al.*, 2012; Ocaña *et al.*, 2012), this might not be the case in all molecular subtypes. We found that in TNBC, activation of the EMT program resulted in a significantly increased proliferation of PT and LNM. This would mean that in TNBC colonization can be triggered by EMT. Results presented by Xu *et al.* indicated that EMT induction in TNBC cancer cell line MDA-MB-231 induces Plasminogen Activator Inhibitor-1 (PAI-1) expression, which increases proliferation of cancer cells (Xu *et al.*, 2018). Unfortunately, we were unable to profile TNBC with a Nanostring panel, thus immunological changes connected with these features could not be assessed. Another limitation of our study is a small sample size, especially for TNBC. Further studies on an extended set of samples are required to investigate the role of EMT in BC molecular subtypes in more detail. We predict that spatial transcriptomics could give more detailed insight into the heterogeneity of EMT status activation in PT/LNM samples and will allow to study the interaction of cancer cells and the immune cells with higher resolution.

To summarize, our results show that in breast cancer EMT activation is connected with progression of luminal PT from non-colonizing (N–) to colonizing stage (N+), and in the TNBC EMT enhances proliferation of cancer cells. Moreover, our data underline the complexity of stroma-related factors in inducing/maintaining EMT in cancer cells at different stages of cancer progression, pointing to a role of antigen presenting cells in supporting mesenchymal phenotype of N– PT.

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Materiały uzupełniające do Publikacji 3

Supplementary Data

TITLE Activation of epithelial-mesenchymal transition process during breast cancer progression – the impact of molecular subtype and stromal composition

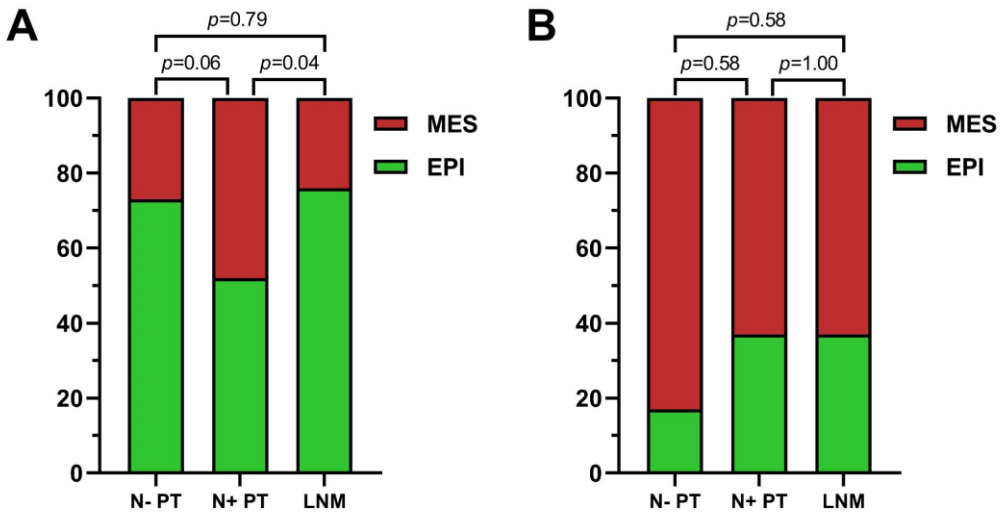

Figure S1. Percentages of epithelial (EPI) and mesenchymal (MES) phenotypes of PT which have not (N-) or have metastasized to the lymph nodes (N+) as well as lymph node metastases themselves (LNM) divided into luminal (panel A) or TNBC molecular subtype (B). Significance levels were values calculated with Pearson's chi-squared or Fisher's exact test.

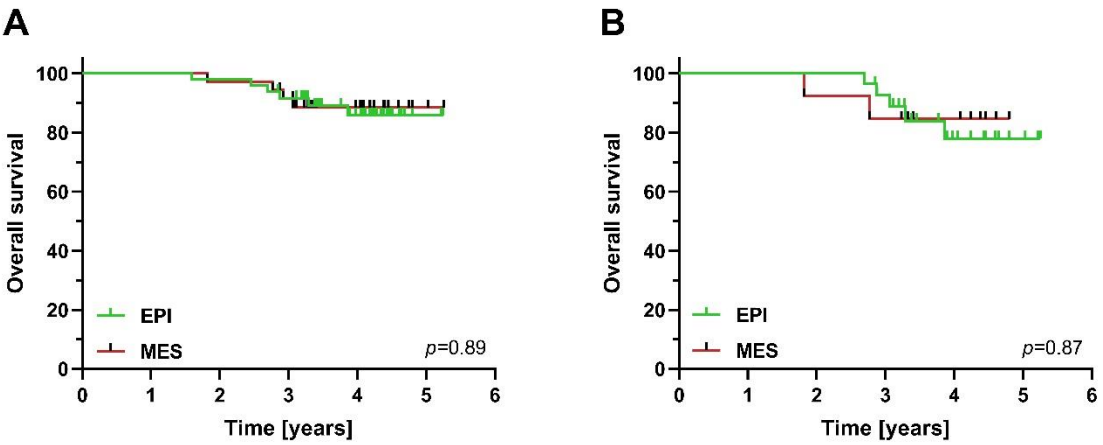

Figure S2. Prognostic significance of EMT phenotypes in primary tumours (A) and lymph node metastases (B) - effect on overall survival of the patients (log-rank test).

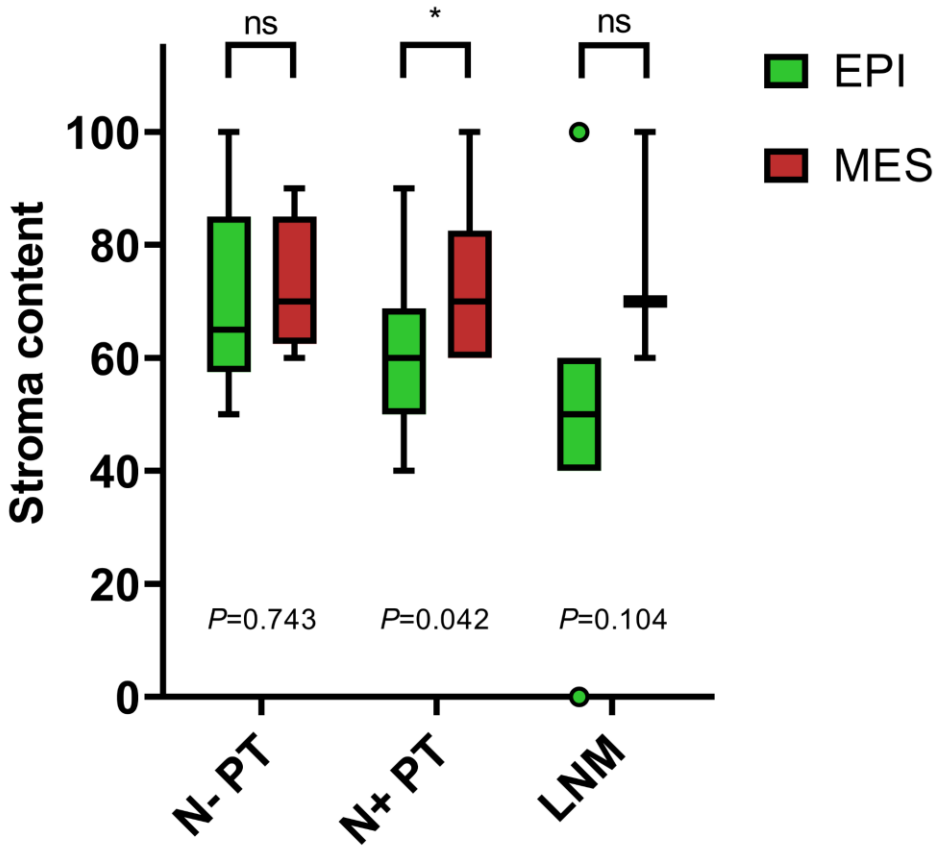

Figure S3. Distribution of stroma content in N- PT, N+ PT and LNM specimens.
The maximum record out of 5 evaluated tissue cores is plotted for each specimen. Differences in median stroma content between EMT groups were analysed with the Mann–Whitney U test; the bars correspond to the interquartile range (IQR), the whiskers cover 1.5 IQR from the median.

Table S1. Clinico-pathological characteristics of the breast cancer patients included in the study.

Variable	Status	Number of cases	%
Age	<50 years	22	25.0
	>50 years	66	75.0
T stage	T1	39	44.3
	T2	43	48.9
	T3	3	3.4
	T4	2	2.3
	Missing data	1	1.1
N stage	N-	43	48.9
	N+	45	51.1
Grade	G1	11	12.5
	G2	46	52.3
	G3	31	35.2
ER	Negative	18	20.5
	Positive	70	79.5
PR	Negative	21	23.9
	Positive	67	76.1
HER2	Negative	65	73.9
	Positive	23	26.1
Molecular subtype	Luminal	74	84.1
	TNBC	14	15.9

Table S2. List of all DEGs in each group of tissues – N- PT, N+ PT and LNM, analysed according to their EMT phenotype.

N- PT				
Gene	Epithelial	Mesenchymal	FC	p-value
CREB1	10.57	10.99	0.42	0.006
EWSR1	12.48	12.20	-0.28	0.012
PRG2	6.52	4.04	**-2.48**	0.018
CSF1	9.17	8.58	-0.59	0.024
IL1R1	9.33	10.16	0.83	0.024
MAP4K2	9.60	9.14	-0.46	0.024
PTGS2	7.75	6.22	**-1.54**	0.024
IFNA7	5.36	3.81	**-1.55**	0.028
ITGA2B	6.29	4.04	**-2.24**	0.029
BST1	8.47	8.81	0.34	0.042
CD164	11.98	13.03	**1.05**	0.042
CXCR4	11.95	12.65	0.71	0.042
FCGR3A	10.67	11.25	0.59	0.042
MAP3K5	10.50	9.95	-0.55	0.042
TNFSF13B	8.18	8.96	0.78	0.042
CXCL3	6.77	5.17	**-1.60**	0.047
N+ PT				
Gene	Epithelial	Mesenchymal	FC	p-value
HLA-DQA1	4.55	12.94	**8.38**	0.013
HLA-DQB1	4.55	11.04	**6.49**	0.016
TNFRSF17	8.58	7.64	-0.94	0.021
LTK	5.83	5.13	-0.70	0.022
POU2AF1	7.62	6.78	-0.84	0.030
CFB	12.66	11.51	**-1.14**	0.036
IFITM1	13.33	12.37	-0.96	0.036
LBP	8.47	5.67	**-2.80**	0.036
ICAM4	6.86	5.46	**-1.40**	0.038
ADA	8.78	8.09	-0.68	0.038
DUSP4	10.78	11.92	**1.15**	0.042
S100A7	9.79	4.58	**-5.21**	0.047
CD47	12.32	11.85	-0.46	0.049
LNM				
Gene	Epithelial	Mesenchymal	FC	p-value
IFITM2	13.48	12.37	**-1.11**	0.006
IKBKB	9.83	10.59	0.76	0.042
NOTCH1	9.67	10.28	0.61	0.042
TANK	11.16	11.30	0.13	0.042

Table S3. List of GO BP and Reactome terms enriched in each list of DEGs - in N- PT, N+ PT and LNM tissues.

N- PT

Category	Term	Count	%	PValue	Genes	List To	Pop Hi	Pop To	Fold Er	Bonfer	Benjar	FDR
GOTERM_BP_DIRECT	GO:0006955~immune response	7	43.75	**9.90E-07**	CD164, MAF	16	421	16792	17.45	**2.31E-04**	**2.31E-04**	**2.31E-04**
GOTERM_BP_DIRECT	GO:0006954~inflammatory response	4	25.00	**4.24E-03**	CSF1, CXCR4	16	379	16792	11.08	6.29E-01	4.69E-01	4.69E-01
GOTERM_BP_DIRECT	GO:0045087~innate immune response	4	25.00	**6.03E-03**	MAP4K2, IFI	16	430	16792	9.76	7.56E-01	4.69E-01	4.69E-01
GOTERM_BP_DIRECT	GO:0045672~positive regulation of osteoclast differentiation	2	12.50	**1.68E-02**	CREB1, CSF1	16	19	16792	110.47	9.81E-01	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0043065~positive regulation of apoptotic process	3	18.75	**2.87E-02**	CREB1, PTGS	16	300	16792	10.50	9.99E-01	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0040018~positive regulation of multicellular organism growth	2	12.50	**3.08E-02**	CREB1, CSF1	16	35	16792	59.97	9.99E-01	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0007257~activation of JUN kinase activity	2	12.50	**3.34E-02**	MAP4K2, M	16	38	16792	55.24	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0030890~positive regulation of B cell proliferation	2	12.50	**3.43E-02**	BST1, TNFSF	16	39	16792	53.82	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0007254~JNK cascade	2	12.50	**4.29E-02**	MAP4K2, M	16	49	16792	42.84	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0006959~humoral immune response	2	12.50	**4.97E-02**	BST1, IFNA7	16	57	16792	36.82	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0030097~hemopoiesis	2	12.50	5.14E-02	CD164, CSF1	16	59	16792	35.58	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0007613~memory	2	12.50	5.40E-02	CREB1, PTGS	16	62	16792	33.85	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0050727~regulation of inflammatory response	2	12.50	5.48E-02	IL1R1, PTGS2	16	63	16792	33.32	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0046330~positive regulation of JNK cascade	2	12.50	5.65E-02	MAP4K2, M	16	65	16792	32.29	1.00E+00	8.97E-01	8.97E-01
REACTOME_PATHWAY	R-HSA-380108~Chemokine receptors bind chemokines	2	12.50	5.70E-02	CXCR4, CXCI	10	59	9075	30.76	9.15E-01	1.00E+00	1.00E+00
GOTERM_BP_DIRECT	GO:0006468~protein phosphorylation	3	18.75	6.12E-02	MAP4K2, CR	16	456	16792	6.90	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0070098~chemokine-mediated signaling pathway	2	12.50	6.16E-02	CXCR4, CXCI	16	71	16792	29.56	1.00E+00	8.97E-01	8.97E-01
GOTERM_BP_DIRECT	GO:0009615~response to virus	2	12.50	9.39E-02	IFNA7, CXCF	16	110	16792	19.08	1.00E+00	1.00E+00	1.00E+00

N+ PT

Category	Term	Count	%	PValue	Genes	List To	Pop Hi	Pop To	Fold Er	Bonfer	Benjar	FDR
GOTERM_BP_DIRECT	GO:0008228~opsonization	2	15.38	**7.13E-03**	CD47, LBP	13	10	16792	258.34	6.33E-01	8.46E-01	8.46E-01
GOTERM_BP_DIRECT	GO:0002504~antigen processing and presentation of peptide or poly	2	15.38	**1.21E-02**	HLA-DQA1,	13	17	16792	151.96	8.18E-01	8.46E-01	8.46E-01
REACTOME_PATHWAY	R-HSA-202430~Translocation of ZAP-70 to Immunological synapse	2	15.38	**2.16E-02**	HLA-DQA1,	10	22	9075	82.50	3.82E-01	1.87E-01	1.87E-01
REACTOME_PATHWAY	R-HSA-202427~Phosphorylation of CD3 and TCR zeta chains	2	15.38	**2.45E-02**	HLA-DQA1,	10	25	9075	72.60	4.21E-01	1.87E-01	1.87E-01
REACTOME_PATHWAY	R-HSA-389948~PD-1 signaling	2	15.38	**2.55E-02**	HLA-DQA1,	10	26	9075	69.81	4.34E-01	1.87E-01	1.87E-01
REACTOME_PATHWAY	R-HSA-202433~Generation of second messenger molecules	2	15.38	**3.52E-02**	HLA-DQA1,	10	36	9075	50.42	5.45E-01	1.93E-01	1.93E-01
GOTERM_BP_DIRECT	GO:0019882~antigen processing and presentation	2	15.38	**3.86E-02**	HLA-DQA1,	13	55	16792	46.97	9.96E-01	1.00E+00	1.00E+00
GOTERM_BP_DIRECT	GO:0050829~defense response to Gram-negative bacterium	2	15.38	**3.86E-02**	LBP, S100A7	13	55	16792	46.97	9.96E-01	1.00E+00	1.00E+00
GOTERM_BP_DIRECT	GO:0060333~interferon-gamma-mediated signaling pathway	2	15.38	**4.96E-02**	HLA-DQA1,	13	71	16792	36.39	9.99E-01	1.00E+00	1.00E+00
GOTERM_BP_DIRECT	GO:0031295~T cell costimulation	2	15.38	5.44E-02	HLA-DQA1,	13	78	16792	33.12	1.00E+00	1.00E+00	1.00E+00
GOTERM_BP_DIRECT	GO:0019886~antigen processing and presentation of exogenous pep	2	15.38	6.38E-02	HLA-DQA1,	13	92	16792	28.08	1.00E+00	1.00E+00	1.00E+00
REACTOME_PATHWAY	R-HSA-216083~Integrin cell surface interactions	2	15.38	8.22E-02	ICAM4, CD4	10	86	9075	21.10	8.48E-01	3.01E-01	3.01E-01
REACTOME_PATHWAY	R-HSA-877300~Interferon gamma signaling	2	15.38	8.40E-02	HLA-DQA1,	10	88	9075	20.63	8.55E-01	3.01E-01	3.01E-01
REACTOME_PATHWAY	R-HSA-202424~Downstream TCR signaling	2	15.38	9.59E-02	HLA-DQA1,	10	101	9075	17.97	8.91E-01	3.01E-01	3.01E-01

LNM

Category	Term	Count	%	PValue	Genes	List To	Pop Hi	Pop To	Fold Er	Bonfer	Benjar	FDR
GOTERM_BP_DIRECT	GO:0035666~TRIF-dependent toll-like receptor signaling pathway	2	50.00	**4.99E-03**	IKBKB, TANI	4	28	16792	299.86	5.84E-01	8.54E-01	8.54E-01
GOTERM_BP_DIRECT	GO:0007249~I-kappaB kinase/NF-kappaB signaling	2	50.00	**1.07E-02**	IKBKB, TANI	4	60	16792	139.93	8.47E-01	8.54E-01	8.54E-01
GOTERM_BP_DIRECT	GO:0009615~response to virus	2	50.00	**1.95E-02**	IKBKB, IFITM	4	110	16792	76.33	9.68E-01	8.54E-01	8.54E-01
GOTERM_BP_DIRECT	GO:0071356~cellular response to tumor necrosis factor	2	50.00	**1.95E-02**	IKBKB, TANI	4	110	16792	76.33	9.68E-01	8.54E-01	8.54E-01
GOTERM_BP_DIRECT	GO:0006955~immune response	2	50.00	7.33E-02	NOTCH1, IFI	4	421	16792	19.94	1.00E+00	1.00E+00	1.00E+00
GOTERM_BP_DIRECT	GO:0045893~positive regulation of transcription, DNA-templated	2	50.00	8.92E-02	IKBKB, NOTC	4	515	16792	16.30	1.00E+00	1.00E+00	1.00E+00

Oświadczenia współautorów do Publikacji 3

Gdańsk, dnia 13.06.2022

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P3]

Markiewicz A, Topa J, Popęda M, Szade J, Skokowski J, Wełnicka-Jaśkiewicz M, Żaczek A

Activation of epithelial-mesenchymal transition process during breast cancer progression – the impact of molecular subtype and stromal composition

Acta Biochim Pol. 2021 Aug 25;68(3):385-392. doi: 10.18388/abp.2020_5719

mój wkład obejmował:

- opracowanie metodologii analizy immunotranskryptomu, w tym analizy bioinformatycznej;
- wykonanie analizy immunotranskryptomu (w tym analiza bioinformatyczna), analizę statystyczną, opracowanie oraz interpretację tych wyników; interpretację wyników analizy histopatologicznej (oznaczenie zawartości podścieliska);
- zarządzanie danymi transkryptomicznymi;
- udział w przygotowaniu manuskryptu;
- udział w przygotowaniu rycin i tabel;
- udział w odpowiedzi na recenzję.

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OŚWIADCZENIE WSPÓŁAUTORA

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mój wkład obejmował:

- opracowanie koncepcji badań;
- opracowanie metodologii analizy danych klinicznych, określania fenotypu EMT oraz plastyczności fenotypowej nowotworu;
- udział w wykonaniu analizy danych klinicznych (określenie fenotypu EMT oraz plastyczności fenotypowej nowotworu na podstawie analizy immunohistochemicznej); analizę statystyczną, opracowanie i interpretację tych wyników; udział w interpretacji wyników analizy immunotranskryptomu;
- udział w przygotowaniu manuskryptu;
- przygotowanie odpowiedzi na recenzję;
- nadzór merytoryczny nad badaniami i przygotowaniem publikacji.

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OŚWIADCZENIE WSPÓŁAUTORA

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mój wkład obejmował:

- udział w wykonaniu analizy danych klinicznych (w tym analiza przeżycia), analizie statystycznej, opracowaniu wyników i ich interpretacji;
- zarządzanie danymi klinicznymi i immunohistochemicznymi;
- udział w przygotowaniu manuskryptu;
- udział w przygotowaniu rycin i tabel;
- udział w odpowiedzi na recenzję.

(podpis)

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Oświadczam, iż w pracy [P3]

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mój wkład obejmował:

- opracowanie metodologii analizy histopatologicznej (oznaczenie zawartości podścieliska);
- wykonanie analizy immunohistochemicznej (oznaczenie oraz ocena ekspresji E-kadheryny, N-kadheryny, wimentyny) oraz analizy histopatologicznej.

(podpis)

Gdańsk, dnia 13.06.2022

Prof. dr hab. Anna J. Żaczek
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OŚWIADCZENIE WSPÓŁAUTORA

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Markiewicz A, Topa J, Popęda M, Szade J, Skokowski J, Wełnicka-Jaśkiewicz M, Żaczek A

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mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- nadzór merytoryczny nad przygotowaniem publikacji;
- pozyskanie środków na badania i kierowanie projektem;
- zapewnienie zaplecza badawczego, w tym niezbędnej infrastruktury;
- koordynację działań projektowych, w tym pozyskania materiału klinicznego, danych klinicznych i histopatologicznych oraz analizy molekularnej.

(podpis)

Publikacja 4 [P4]

Popeda M, Markiewicz A, Stokowy T, Szade J, Niemira M, Kretowski A, Bednarz-Knoll N, Zaczek AJ

Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients

Sci Rep. 2021 Mar 3;11(1):5097. doi: 10.1038/s41598-021-84568-0

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Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients

Marta Popeda[1], Aleksandra Markiewicz[1], Tomasz Stokowy[2], Jolanta Szade[3], Magdalena Niemira[4], Adam Kretowski[4], Natalia Bednarz-Knoll[1] & Anna J. Zaczek[1]✉

Immune system plays a dual role in cancer by either targeting or supporting neoplastic cells at various stages of disease, including metastasis. Yet, the exact immune-related transcriptome profiles of primary tumours (PT) and lymph node metastases (LNM) and their evolution during luminal breast cancer (BCa) dissemination remain undiscovered. In order to identify the immune-related transcriptome changes that accompany lymphatic spread, we analysed PT-LNM pairs of luminal BCa using NanoString technology. Decrease in complement C3—one of the top-downregulated genes, in LNM was validated at the protein level using immunohistochemistry. Thirty-three of 360 analysed genes were downregulated (9%), whereas only 3 (0.8%) upregulated in LNM when compared to the corresponding PT. In LNM, reduced expression was observed in genes related to innate immunity, particularly to the complement system (*C1QB*, *C1S*, *C1R*, *C4B*, *CFB*, *C3*, *SERPING1* and *C3AR1*). In validation cohort, complement C3 protein was less frequently expressed in LNM than in PT and it was associated with worse prognosis. To conclude, local expression of the complement system components declines during lymphatic spread of non-metastatic luminal BCa, whilst further reduction of tumoral complement C3 in LNM is indicative for poor survival. This points to context-dependent role of complement C3 in BCa dissemination.

The metastatic disease remains the leading cause of cancer-related deaths. Metastasis is a multistep process that involves the action of both tumour microenvironment (TME), comprising immune cells and stromal components, and cancer cells. The current opinion states that cancer cells tend to spread either via lymph or blood, reaching their specific final destination—lymph nodes or distant organs[1]. In breast cancer (BCa), lymph nodes are the first site to be colonized through the lymphatic route, usually much earlier than the distant sites reached via haematogenic route. Recent research has demonstrated that distant metastases of BCa may be seeded from the metastatic foci in lymph nodes[2–4]. As lymph nodes play a key role in immune response, they may also contribute to the selection of the immune-evading phenotype of cancer cells, thus driving further metastatic spread. Still, the transcriptional changes that accompany the dissemination process remain unknown.

The immune system, although originally developed for defence against pathogens, is a key player in cancer development and progression. The interaction between the tumour and surrounding immune cells is constant and complex, leading either to inhibition or stimulation of tumour growth, as included in the hallmarks of cancer by Hanahan and Weinberg[5]. In contrary to common knowledge, luminal breast tumours have recently been demonstrated to exhibit heterogeneous immunogenicity reflected by distinct patterns of immune gene expression[6,7].

Thus, in this study we aimed to explore the changes of immune-related transcriptome indicative for metastatic colonization in luminal BCa. We compared data on 360 immune-related genes expression in matched pairs of primary breast tumours (PT) and lymph node metastases (LNM). To compensate for the physiological differences between breast and lymph node tissue, we incorporated a healthy background normalization step based on normal tissue expression data from GeneCards database. The selected transcriptional changes were subsequently validated at the protein level using immunohistochemical (IHC) staining.

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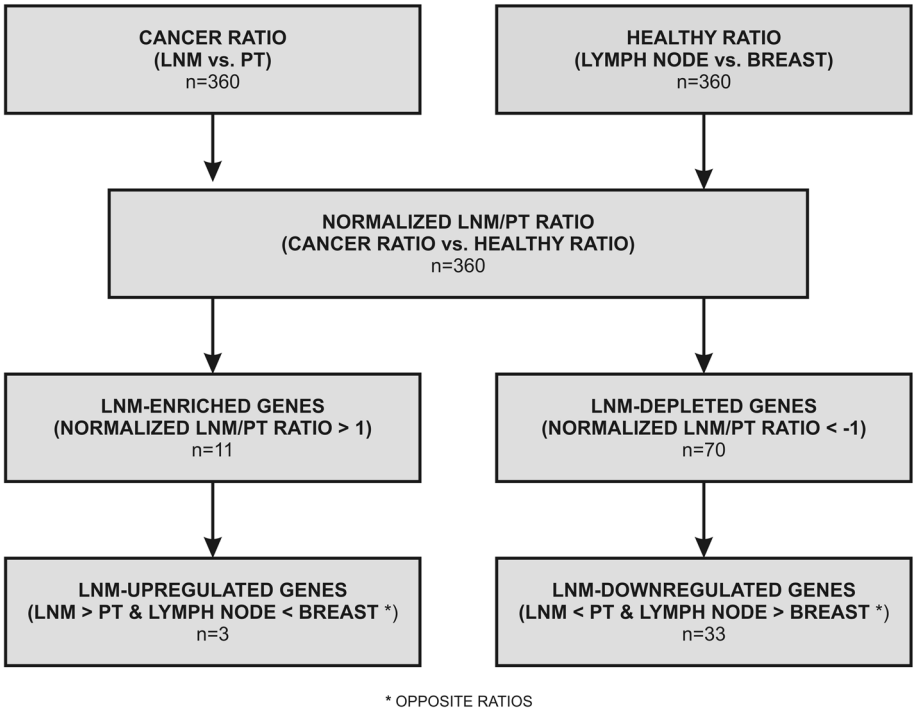

Figure 1. General flow of data analysis. 360 genes were analysed in 11 matched pairs of PT and LNM tissues (CANCER; NanoString data) with a healthy-background normalization based on expression data for healthy breast and lymph node tissues (HEALTHY; Illumina Body Map data); for each gene, normalized LNM/PT ratio was calculated for each matched LNM-PT pair separately, and the median of all normalized LNM/PT ratios was employed in further analyses.

Results

Immune-related transcriptomic changes during metastatic colonization in luminal BCa. *LNM vs. LYMPH NODE comparison.* Owing to the physiological transcriptome differences between lymph node and breast tissue, we decided to include a healthy-background-normalization step in our analysis (Fig. 1). Using expression data for lymph nodes (LYMPH NODE) and breast tissue (BREAST) from GeneCards database (GTEx project data[8]), we calculated median normalized LNM/PT ratios for 360 genes linked with the immune system.

Here we observed a substantial disproportion in the number of genes that were enriched or depleted in LNM in comparison to healthy lymph node. Eleven of 360 genes demonstrated increased expression in LNM, while a decrease was observed in 70 genes (Supplementary Table S1). LNM-enriched genes were primarily breast, breast cancer or pro-metastatic markers, while the LNM-depleted genes mainly contributed to innate immune response. These observations showed that in the LNM, the healthy lymphatic tissue—physiologically abundant in immune-related transcripts—was replaced by cancer, which putatively induced changes in stromal cells (TME) and was connected with decreased innate immune response.

LNM versus PT comparison. In the next step, we looked for transcriptional changes from PT to LNM. Based on the opposite ratios of gene expression levels in LYMPH NODE/BREAST and LNM/PT, we selected LNM-upregulated genes from LNM-enriched genes (3/11) and LNM-downregulated genes from LNM-depleted genes (33/70) (Supplementary Table S1). All LNM-upregulated and top10 LNM-downregulated genes are depicted in Fig. 2.

Considering their biological role, 3 LNM-upregulated genes (*ATG10*, *GATA3* and *S100B)* are potential markers of aggressive phenotype and increased metastatic potential of cancer cells. On the other hand, 33 LNM-downregulated genes are mainly associated with innate immunity, in particular, with the complement cascade, as revealed by the functional annotation analysis (Fig. 3, Supplementary Table S2).

Complement component 3 (C3) protein expression decreases during metastasis—IHC validation. Downregulation of transcripts related to complement cascade in LNM, when compared to corresponding PT, indicates that local expression of complement might play a distinct role in PT and LNM. For that reason we decided to validate our transcriptional findings at the protein level, focusing on the central element of all activation cascades—complement component 3 (C3). Using IHC staining, we evaluated C3 expression in the larger cohort of luminal BCa patient (n = 79) with both positive (N+, n = 43) and negative (N−, n = 36) nodal involvement status, including the samples analysed with NanoString technology.

The C3 staining was informative for 36 of 36 N− PT, 42 of 43 N+ PT and 36 of 43 LNM specimens, resulting in 35 matched PT-LNM pairs. We evaluated stromal, tumoral and overall (i.e. combined C3 expression in both

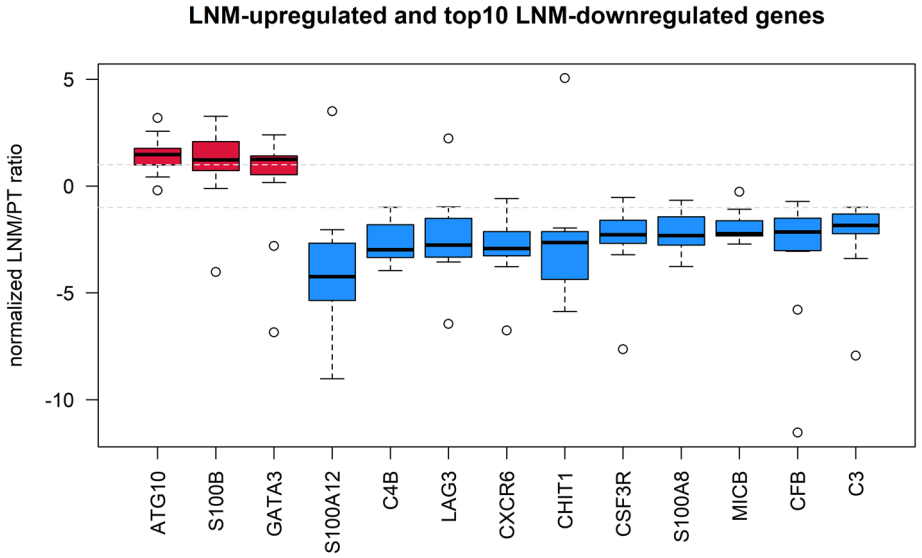

Figure 2. Distribution of normalized LNM/PT ratios of LNM-upregulated and -downregulated genes. Genes were classified as LNM-upregulated (red) and LNM-downregulated (blue; top10 out of 33 genes are depicted) based on the median normalized LNM/PT ratio and opposite LYMPHNODE/BREAST and median matched LNM/PT ratios. Grey dashed lines represent the cut-off for LNM-enrichment (1) and LNM-depletion (−1).

tumour cells and stroma) C3 expression status and dichotomized it as negative or positive based on staining intensity. In 11 matched PT-LNM pairs for which both NanoString and IHC data were available, mRNA levels and protein status tended to correlate when C3 protein was evaluated in tumour cells, however, did not reach the statistical significance due to the low number of samples (Supplementary Figure S2). In addition, in N+ patients, tumoral C3 was significantly decreased in LNM in comparison to PT (Fig. 4A), while no difference in C3 presence was noted in the stromal compartment (Fig. 4B). Combined stromal and tumoral assessment (overall status) showed a trend toward reduced C3 positivity in LNM (Fig. 4C). Similar tendencies were also observed when N+ and N− patients were analysed together (Fig. 4D–F). Representative images of tumoral C3 staining are presented in Fig. 4G. Of note, C3 expression in tumour cells of PT did not correlate with any clinical features, including stage, T status, grade and CTC status (data not shown). Intriguingly, loss of C3 expression in LNM tumour cells was associated with shorter 3-year overall survival (Supplementary Figure S3).

Discussion

Interactions between tumour cells and TME remain still unexplored, particularly in the context of cancer progression. In the current study, we focused on the most common molecular subtype of BCa—luminal cancers, where we demonstrate that the immune-related transcriptome changes occurring between primary tumour (PT) and lymph node metastasis (LNM) are mainly related to innate immunity, i.e. complement pathway.

In the exploratory group consisting of matched PT and LNM from 11 non-metastatic luminal BCa patients, 9% of transcripts were downregulated, whereas only 0.8% of transcripts were upregulated in LNM when compared to PT. According to the literature, all 3 identified LNM-upregulated genes, i.e. *ATG10*, *GATA3* and *S100B*, are considered markers of breast cancer cells and their invasive potential[9–13]. *ATG10* encodes a protein involved in autophagy cascade known to support or even induce metastatic process[9,10]. *GATA3* codes for a transcription factor that is particularly abundant in luminal epithelial cells and directly correlates with *ESR1* expression, thus is considered a marker of luminal breast cancer[11,12], whereas *S100B* belongs to a family of genes coding for calcium-binding inflammatory proteins, regulators of p53, and might be involved in acid-induced EMT of ER-positive breast cancer cells in vitro[14]. The above-described enrichment in breast cancer and metastasis-associated transcripts proves that our algorithm is capable of adjusting the detected changes to the original characteristics of compared tissues, presumably due to the appropriate application of healthy tissue normalization step. On the other hand, the genes that were downregulated in LNM in comparison to PT were mainly associated with innate immunity, in particular complement system pathways. Interestingly, the identified complement-related genes are implicated in the early steps of the cascade, including all 3 pathways of its activation—*C1QB*, *C1S*, *C1R*, *C4B*, *CFB* and *C3*, regulation—*SERPING1*, as well as its effector mechanisms—*C3AR1* (coding for receptor expressed on the surface of a variety of immune cells).

The complement system is known to play a dual role in cancer. As a fundamental part of the innate immunity, it is capable of targeting cancer cells and managing the immune response against the tumour. On the other hand, as a potent pro-inflammatory mechanism, the complement system is thought to substantially contribute to tumour growth by generating chronic inflammation state that facilitates mobilization of immune suppressor cells[15] and supports angiogenesis[16]. In general, proteins comprising the complement system are synthesized in the liver and then released into plasma, resulting in the extracellular body compartments being the main environment for their interaction and cascade activation[17–20]. Nonetheless, a growing body of evidence suggests

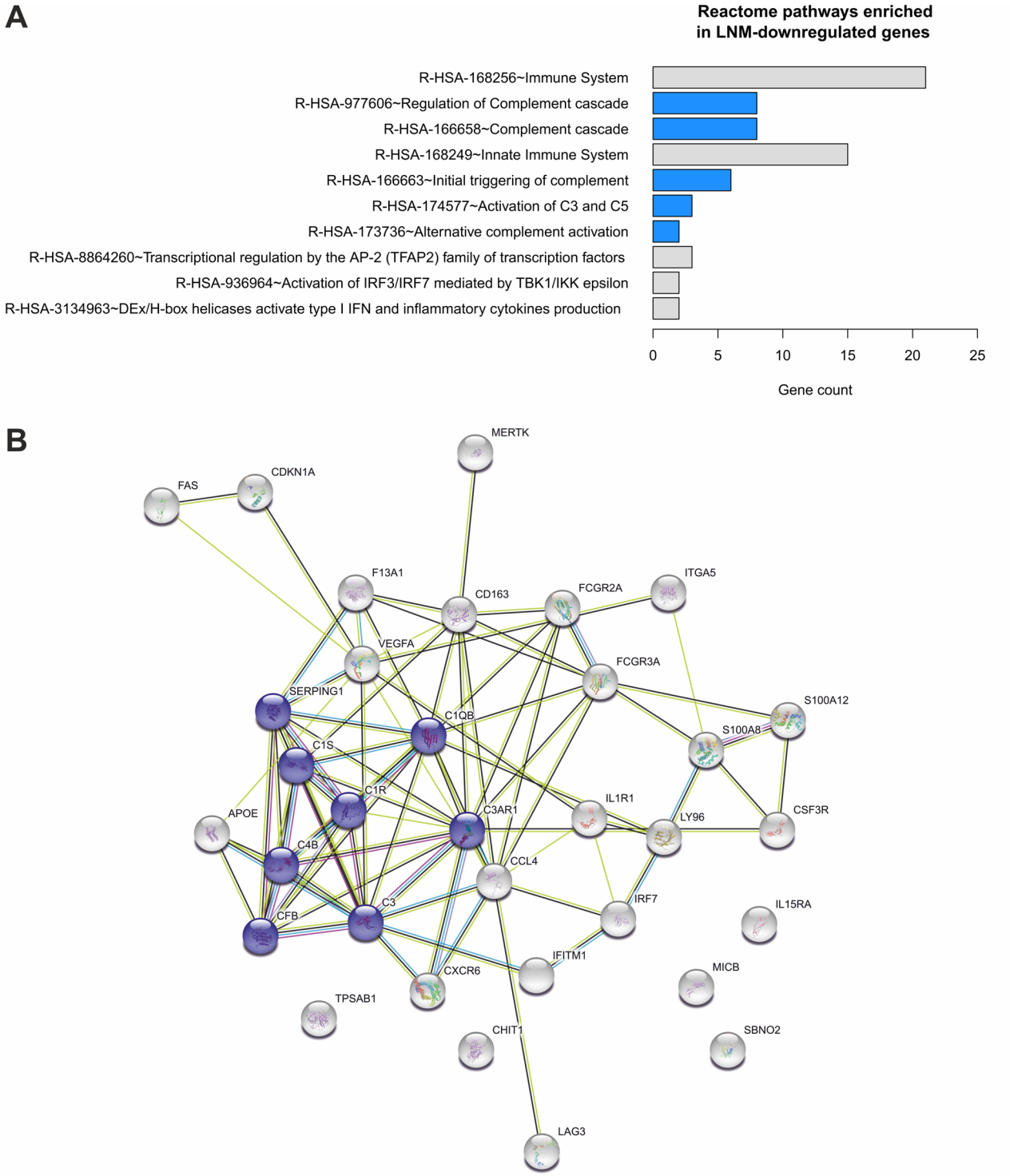

Figure 3. LNM-downregulated genes are associated with the complement system. (**A**) ConsensusPathDB over-representation analysis [pathways ordered according to −log 10(FDR adjusted *p* value), complement-related pathways marked in blue] and (**B**) STRING protein interaction analysis (complement-related protein products marked in purple).

that the individual components of the complement pathway may also be produced within a tumour by both cancer and stromal cells. This is also supported by our data on high expression of C3 protein in tumour cells in BCa. Still, the locally produced complement proteins are thought to have a non-canonical function and act in a context-dependent manner, which merits further exploration[18,21,22].

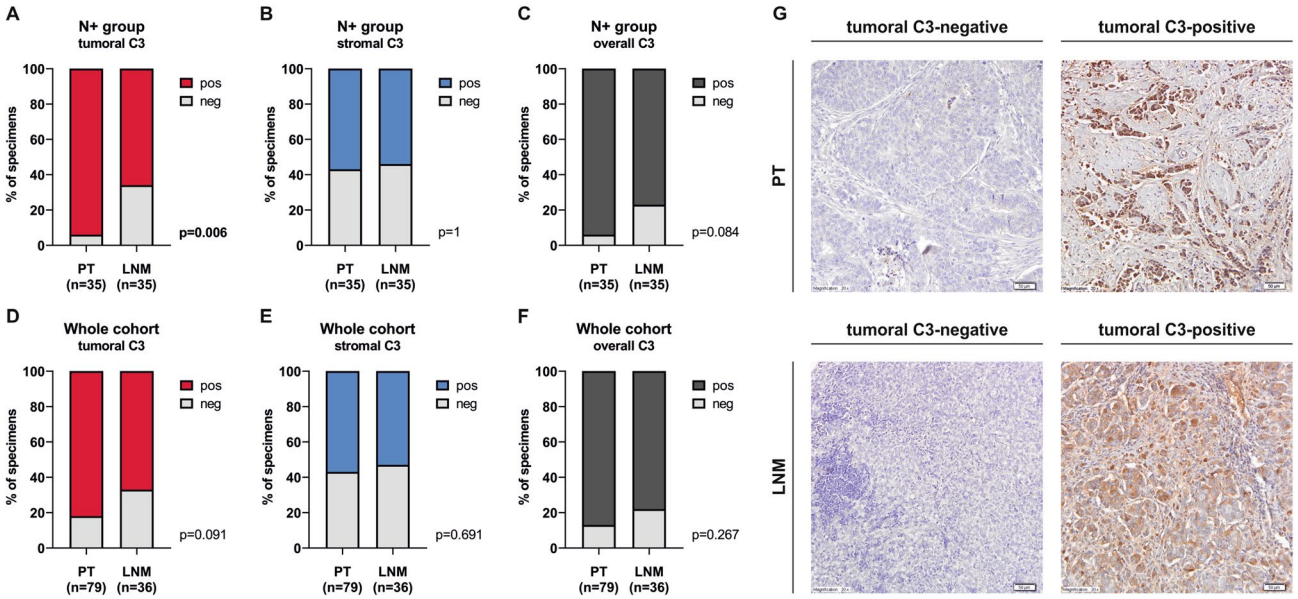

Figure 4. C3 protein expression is reduced during the lymphatic spread of BCa. N+ group evaluated for tumoral (**A**), stromal (**B**) and overall (**C**) C3 expression in PT and LNM; whole cohort evaluated for tumoral (**D**), stromal (**E**) and overall (**F**) C3 expression in PT and LNM; number of informative specimens is presented in Supplementary Figure S4; distribution was estimated with Fisher's exact test. Representative images of C3 immunohistochemical staining in PT and LNM (**G**); presented tumoral C3-positive tissues also show a weak stromal C3 expression.

The association between the complement system and tumour dissemination has been broadly studied at the mRNA level. Several reports show that in the absence of EMT-promoting signals, C3 could enhance metastasis of epithelial cancer cells by loosening cellular junctions[23], modulating vascularization and endothelial cells function[24] or promoting survival of cancer cells via increasing their interactions with platelets[25,26]. On the other hand, the ovarian cancer mouse model provided evidence on the correlation between mRNA *C3* expression and lymphatic vasculature[24]. Finally, the complement system also appeared to facilitate early stages of metastasis via modulation of cell adherence in several cancer types[27].

In breast cancer, local expression of complement inhibitors was reported and perceived as a mechanism of evading immune response and cytocidal complement function. Namely, in primary breast tumours the expression of factor I and CD46 correlated with larger tumour size, lower differentiation index, and poor prognosis[28,29]. Moreover, in animal models of BCa, two groups reported the role of complement in premetastatic niche formation in the lungs[30,31]. Nevertheless, there is little clinical material-derived evidence on the exact role of the complement system in breast cancer dissemination. Still, a recent report by Chatterjee et al.[32] has comprehensively summarized gene expression changes from healthy to metastatic (sentinel and non-sentinel) lymph node.

In our study, the reduction in local mRNA expression of C3—the central node of all complement activation cascades—in lymph node metastases was confirmed at the protein level. To the best of our knowledge, this is the first evidence for the association between C3 protein expression in breast cancer and metastatic colonization of lymph nodes. By far only one study by Chen and colleagues examined the relationship between the C3 protein and TNM staging and nodal involvement in pancreatic cancer, providing negative results[33]. The report by Vadrevu et al. also demonstrated that in BCa patients the complement proteins, including C3, are produced in both metastatic and metastasis-free nodes, yet the expression level in colonized lymph nodes was substantially higher. Still, the authors conclude that the complement system affects the metastatic process in a context-dependent manner[30]. This is in line with the outcomes of a comprehensive TCGA data analysis by Roumenina et al., investigating the complement system, TME and their prognostic properties in 30 cancer types. Based on survival analysis according to the level of complement-related transcriptome, BCa was classified as a cancer type with uncertain complement significance[18], perhaps due to the distinct biology of the molecular subtypes of breast tumours. This provides a rationale for further exploration of the complement system role in BCa biology.

One of the limitations of our study was the small size of the exploration group (n = 11) in NanoString analysis. To compensate for that we performed an immunohistochemical validation of selected results on a larger cohort of patients (n = 79), proving the accuracy of our transcriptomic results. Another limitation was the application of NGS (RNA-seq) data on healthy tissues transcriptome for the healthy background normalization step. Since healthy tissue material is rarely included in high-throughput gene expression studies due to both its limited availability and high cost of the analysis, no compatible NanoString data set was available for use in public databases. A growing number of studies proves that NanoString and RNA-seq are compatible for gene expression analysis, both at the single gene and pathway level[34–37]. According to Zhang et al., NanoString and RNA-seq show the highest correlation coefficient among all available transcriptomic methods[38]. Nonetheless, aware of the limitations of comparing data from two different platforms, we did not normalize our NanoString cancer data

Parameter	Status	Whole cohort (n = 79)		NanoString group (n = 11)	
		n	%	n	%
Clinical stage	I	22	28	0	0
	II	41	52	6	55
	III	16	20	5	45
T	1	33	42	1	9
	2	41	52	4	36
	3	3	4	6	55
	4	2	3	0	0
N	Negative	36	46	0	0
	Positive	43	54	11	100
CTC	Negative	44	56	6	55
	Epithelial	13	16	0	0
	Mesenchymal	9	11	5	45
	NA	13	16	0	0
Grade	1	14	18	1	9
	2	45	57	4	36
	3	20	25	6	55
Histological type	NST	68	86	11	100
	Other	11	14	0	0
ER status	Negative	4	5	1	9
	Positive	75	95	10	91
PR status	Negative	7	9	0	0
	Positive	72	91	11	100
HER2 status	Negative	57	72	8	73
	Positive	22	28	3	27
Molecular subtype	lumA	31	39	2	18
	lumB HER2-	26	33	6	55
	lumB HER2+	22	28	3	27

Table 1. Clinicopathological characteristics of the cohort.

using the NGS results for healthy tissues; instead, to minimize platform-specific differences, normalization was conducted within the same platform (Fig. 1).

To conclude, based on our findings we propose that the complement system potentially contributes to breast cancer lymphatic spread. We observed reduced mRNA expression of complement system genes in colonized lymph nodes when compared to corresponding primary tumours. Importantly, we also demonstrate that protein expression of complement C3 in cancer cells decreases in the course of metastatic spread and its further decrease in lymph node metastases is linked with patients poor prognosis. Consequently, we hypothesize that in non-metastatic luminal breast cancer patients the local PT expression of complement system-related genes may facilitate their invasion and metastasis, and eventually become decreased to alleviate immune system reaction when the cells reach the lymph node. Due to its potential role in shaping the aggressive phenotype of luminal breast tumours, the complement system appears to be a potential target for cancer treatment and thus merit further studies.

Methods

Patients. The study group consisted of 79 non-metastatic luminal BCa patients staged I–III, who underwent surgical treatment at the Medical University Hospital in Gdansk between 2011 and 2013. The study was approved by the Ethical Committee of the Medical University of Gdansk (NKBBN 94/2017) and informed consent was collected from all participants. All experiments were conducted in accordance with the Declaration of Helsinki, REMARK[39] and STROBE[40]. Patients were characterized by different clinicopathological parameters, including nodal involvement and CTC status, as described previously[41] and summarised in Table 1. Transcriptome analysis covered archival FFPE samples of PT and 11 LNM from 11 selected N+ patients for whom matched PT-LNM pairs were available. The algorithm of patients/sample selection is depicted in Supplementary Figure S4.

nCounter transcriptome profiling of primary breast cancer and corresponding lymph node metastasis fragments. Total RNA was isolated from archival FFPE blocks using RNeasy Mini Kit (Qiagen) and the expression of 730 target genes was evaluated with nCounter PanCancer Immune Profiling Panel (NanoString Technologies), as reported previously[42].

For each analysed sample, background correction and normalization against global mean were performed as described[42], using nSolver 4.0 software (NanoString Technologies). PT and LNM samples were normalized together. In brief, the background level was estimated by thresholding over the mean plus 2 standard deviations of the negative control counts. Subsequently, the data were normalized according to the global mean of the counts of positive controls and 4 most stably expressed housekeeping genes—*ABCF1*, *EDC3*, *HDAC3*, and *CNOT4*. The negative and positive control probes were included in the assay. Following normalization, low-expression genes (log2 mean count in all samples < 6) were excluded, leaving 593 target genes for analysis.

Healthy tissues transcriptome profiles from GeneCards database. Illumina Body Map expression data for normal lymph node (LYMPH NODE) and normal breast tissue (BREAST) generated within the Genotype-Tissue Expression (GTEx) project[8] and deposited in the GeneCards database[43] were obtained as a courtesy of Weizmann Institute of Science. 578 out of 593 genes expressed in the NanoString data were present in the Illumina Body Map dataset. Low-expression genes (log2 FPKM in each normal tissues < 5) were excluded, leaving 360 target genes for analysis.

Transcriptome data analysis. Analysis was performed for 360 genes expressed in both the NanoString and Illumina Body Map datasets (as depicted in Fig. 1, genes listed in Supplementary Table S3). The NanoString dataset comprised 11 matched LNM-PT pairs of BCa samples, whereas the Illumina Body Map dataset included the healthy control tissues—LYMPH NODE and BREAST. In brief, all data were log2 transformed and a log2 LYMPH NODE/BREAST (HEALTHY) ratio was calculated for each gene using Illumina Body Map data. In parallel, a matched log2 LNM/PT (CANCER) ratio was calculated for each patient using NanoString data. Subsequently, the HEALTHY ratio was subtracted from the matched CANCER ratio, giving a healthy background-normalized log2 LNM/PT ratio (further referred to as normalized LNM/PT ratio) for each patient. Eventually, the median normalized LNM/PT ratio was calculated for each gene based on the data of the whole cohort.

Genes with median normalized LNM/PT ratio > 1 were considered LNM-enriched compared to healthy lymph node (Supplementary Figure S5A). Genes with median normalized LNM/PT ratio < −1 were considered LNM-depleted compared to healthy lymph node (Supplementary Figure S5B). LNM-enriched/depleted genes were then analysed for changes in gene expression level from PT to LNM in reference to the healthy background. Genes with LYMPH NODE < BREAST and median normalized LNM > PT expression tendencies were considered LNM-upregulated in comparison to PT (Supplementary Figure S5C), while genes with LYMPH NODE > BREAST and median normalized LNM < PT expression tendencies were considered LNM-downregulated in comparison to PT (Supplementary Figure S5D).

Immunohistochemical evaluation of complement C3 protein. Tissue microarrays (TMA) comprising five 1-mm diameter tumour samples per each patient were prepared as previously described[44]. In brief, to detect C3, TMA sections were deparaffinised and treated with citrate buffer (pH 6, Dako) for 10 min and Peroxidase-Blocking Solution (Dako) for 5 min. The sections were incubated for 1 h at RT with polyclonal rabbit anti-C3 antibody (NBP1-32080, NOVUS Biologicals) diluted 1:250, envisioned by EnVision Kit, Rabbit/Mouse (Dako) and counterstained with haematoxylin (Sigma Aldrich). All tumour samples were evaluated in both tumour cells and surrounding stroma. Intensity of the staining and its semi-quantitative presence was documented. Staining was categorized based on the intensity as negative (i.e. no or weak expression), or positive (i.e. moderate to strong expression), and the tumour samples were scored for stromal, tumoral and overall (i.e. tumour cells and stroma) C3 expression. For each specimen, maximum record out of all examined and informative tumour samples was assigned for further analysis.

Statistical analysis. Data were analysed and visualized using R computing environment (3.6.1)[45] and GraphPad Prism 8 (GraphPad Software) licensed for Medical University of Gdańsk. Differences in the distribution of C3 expression status were estimated with Fisher's exact test. Association between tumoral C3 and overall survival was evaluated using log-rank test. Statistical significance was inferred for p values ≤ 0.05.

Selected genes were functionally annotated with Reactome pathways using over-representation analysis tool by ConsensusPathDB[46]. Interactions between protein products of selected genes were visualized using STRING v11[47].

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on request.

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Author contributions

Conceptualization—N.B.K., A.M., M.P. and A.J.Z.; Methodology—N.B.K., A.K., M.N., M.P., T.S. and J.S.; Formal analysis—A.M., M.P. and T.S.; Investigation—N.B.K., A.M., M.N. and M.P.; Resources—N.B.K., A.K., M.N. and A.J.Z.; Data curation—M.P. and T.S.; Original draft preparation—A.M. and M.P.; Draft review and editing—all authors; Visualization—M.P. and T.S.; Supervision—N.B.K., A.M. and A.J.Z.; Project administration—A.J.Z.; Funding acquisition—A.J.Z.

Competing interests

The authors declare no competing interests.

Additional information

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Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients

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mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- opracowanie metodologii analizy immunotranskryptomu, w tym analizy bioinformatycznej; optymalizację metodologii analizy immunohistochemicznej;
- wykonanie analizy immunotranskryptomu (przygotowanie materiału tkankowego do analizy metodą nCounter, ekstrakcja RNA; analiza bioinformatyczna – dane własne, dane z projektu GTex) oraz analizy immunohistochemicznej (oznaczenie oraz ocena ekspresji białka C3); analizę statystyczną, opracowanie wyników i ich interpretację;
- zarządzanie danymi;
- przygotowanie manuskryptu;
- przygotowanie rycin i tabel;
- przygotowanie odpowiedzi na recenzję.

Marta Popęda

(podpis)

Gdańsk, dnia 9/6/2022

dr Aleksandra Markiewicz
Zakład Onkologii Translacyjnej
Gdański Uniwersytet Medyczny

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P4]

Popeda M, Markiewicz A, Stokowy T, Szade J, Niemira M, Kretowski A, Bednarz-Knoll N, Zaczek AJ

Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients

Sci Rep. 2021 Mar 3;11(1):5097. doi: 10.1038/s41598-021-84568-0

mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- udział w interpretacji wyników analizy immunotranskryptomu (dane własne, dane z projektu GTex);
- redakcję manuskryptu;
- udział w odpowiedzi na recenzję;
- nadzór merytoryczny nad analizą immunotranskryptomu.

Aleksandra Markiewicz
(podpis)

Gdańsk, dnia 09.06.2022

lek. Jolanta Szade
Katedra i Zakład Patomorfologii
Gdański Uniwersytet Medyczny

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P4]

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Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients

Sci Rep. 2021 Mar 3;11(1):5097. doi: 10.1038/s41598-021-84568-0

mój wkład obejmował:

- nadzór merytoryczny nad optymalizacją metodologii analizy immunohistochemicznej (dostarczenie tkanek kontrolnych).

(podpis)

Szade

Gdańsk, dnia 13.06.2022

dr Natalia Bednarz-Knoll
Zakład Onkologii Translacyjnej
Gdański Uniwersytet Medyczny

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P4]

Popeda M, Markiewicz A, Stokowy T, Szade J, Niemira M, Kretowski A, Bednarz-Knoll N, Zaczek AJ

Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients

Sci Rep. 2021 Mar 3;11(1):5097. doi: 10.1038/s41598-021-84568-0

mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- optymalizację metodologii analizy immunohistochemicznej;
- udział w analizie immunohistochemicznej (oznaczenie oraz ocena ekspresji białka C3) oraz interpretacji wyników;
- udział w odpowiedzi na recenzję;
- nadzór merytoryczny nad analizą immunohistochemiczną.

Natalia Bednarz-Knoll
(podpis)

Gdańsk, dnia 13.06.2022

Prof. dr hab. Anna J. Żaczek
Zakład Onkologii Translacyjnej
Gdański Uniwersytet Medyczny

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż w pracy [P4]

Popeda M, Markiewicz A, Stokowy T, Szade J, Niemira M, Kretowski A, Bednarz-Knoll N, Zaczek AJ

Reduced expression of innate immunity-related genes in lymph node metastases of luminal breast cancer patients

Sci Rep. 2021 Mar 3;11(1):5097. doi: 10.1038/s41598-021-84568-0

mój wkład obejmował:

- udział w opracowaniu koncepcji badań;
- redakcję manuskryptu;
- udział w odpowiedzi na recenzję;
- nadzór merytoryczny nad badaniami;
- pozyskanie środków na badania i kierowanie projektem;
- zapewnienie zaplecza badawczego, w tym niezbędnej infrastruktury;
- koordynację działań projektowych, w tym pozyskania materiału klinicznego, danych klinicznych i histopatologicznych oraz analizy molekularnej.

(podpis)

Wykaz osiągnięć

Publikacje niewchodzące w skład rozprawy doktorskiej (łączna liczba: 18)

- Nagel A, **Popeda M**, Muchlinska A, Sadej R, Szade J, Zielinski J, Skokowski J, Niemira M, Kretowski A, Markiewicz A, Zaczek AJ. ERα36-High Cancer-Associated Fibroblasts as an Unfavorable Factor in Triple-Negative Breast Cancer. Cancers (Basel). 2022 Apr 15;14(8):2005. doi: 10.3390/cancers14082005. IF: 6,639
- Zapała Ł, Kunc M, Sharma S, Pęksa R, **Popęda M**, Biernat W, Radziszewski P. Evaluation of PD-L1 (E1L3N, 22C3) expression in venous tumor thrombus is superior to its assessment in renal tumor in predicting overall survival in renal cell carcinoma. Urol Oncol. 2022 Mar 25:S1078-1439(22)00035-7. doi: 10.1016/j.urolonc.2022.02.001. IF: 3,498
- Nastały P, Smentoch J, **Popęda M**, Martini E, Maiuri P, Żaczek AJ, Sowa M, Matuszewski M, Szade J, Kalinowski L, Niemira M, Brandt B, Eltze E, Semjonow A, Bednarz-Knoll N. Low Tumor-to-Stroma Ratio Reflects Protective Role of Stroma against Prostate Cancer Progression. J Pers Med. 2021 Oct 26;11(11):1088. doi: 10.3390/jpm11111088. IF: 4,945
- Kunc M, **Popęda M**, Biernat W, Senkus E. Lost but Not Least-Novel Insights into Progesterone Receptor Loss in Estrogen Receptor-Positive Breast Cancer. Cancers (Basel). 2021 Sep 23;13(19):4755. doi: 10.3390/cancers13194755. IF: 6,639
- Supernat A, **Popęda M**, Pastuszak K, Best MG, Grešner P, Veld SI', Siek B, Bednarz-Knoll N, Rondina MT, Stokowy T, Wurdinger T, Jassem J, Żaczek AJ. Transcriptomic landscape of blood platelets in healthy donors. Sci Rep. 2021 Aug 3;11(1):15679. doi: 10.1038/s41598-021-94003-z. IF: 4,379
- Pęksa R, Kunc M, **Popęda M**, Piątek M, Bieńkowski M, Żok J, Starzyńska A, Perdyan A, Sowa M, Duchnowska R, Biernat W. Combined Assessment of Immune Checkpoint Regulator VISTA on Tumor-Associated Immune Cells and Platelet-to-Lymphocyte Ratio Identifies Advanced Germ Cell Tumors with Higher Risk of Unfavorable Outcomes. Cancers (Basel). 2021 Apr 7;13(8):1750. doi: 10.3390/cancers13081750. IF: 6,639
- Wiśniewski K, **Popęda M**, Tomasik B, Bieńkowski M, Bobeff EJ, Stefańczyk L, Tybor K, Hupało M, Jaskólski DJ. The Role of Urine F2-isoprostane Concentration in Delayed Cerebral Ischemia after Aneurysmal Subarachnoid Haemorrhage - A Poor Prognostic Factor. Diagnostics (Basel). 2020 Dec 22;11(1):5. doi: 10.3390/diagnostics11010005. IF: 3,706
- Nastały P, Stoupiec S, **Popęda M**, Smentoch J, Schlomm T, Morrissey C, Żaczek AJ, Beyer B, Tennstedt P, Graefen M, Eltze E, Maiuri P, Semjonow A, Pantel K, Brandt B, Bednarz-Knoll N. EGFR as a stable marker of prostate cancer dissemination to bones. Br J Cancer. 2020 Dec;123(12):1767-1774. doi: 10.1038/s41416-020-01052-8. IF: 7,640
- Kunc M, **Popęda M**, Niemira M, Szałkowska A, Bieńkowski M, Pęksa R, Łacko A, Radecka BS, Braun M, Pikiel J, Litwiniuk M, Pogoda K, Iżycka-Świeszewska E, Krętowski A, Żaczek AJ, Biernat W, Senkus-Konefka E. microRNA Expression Profile in Single Hormone Receptor-Positive Breast Cancers is Mainly Dependent on HER2 Status-A Pilot Study. Diagnostics (Basel). 2020 Aug 20;10(9):617. doi: 10.3390/diagnostics10090617. IF: 3,706
- Bieńkowski M, Pęksa R, **Popęda M**, Kołaczkowska M, Frankiewicz A, Żaczek AJ, Gruchała M, Biernat W, Siondalski P. Liquid biopsy for minimally invasive heart transplant monitoring: a pilot study. J Clin Pathol. 2020 Aug;73(8):507-510. doi: 10.1136/jclinpath-2019-205926. IF: 3,411

- Janik K, Och W, **Popeda M**, Rosiak K, Peciak J, Rieske P, Kulbacki K, Szostak B, Parda A, Stoczynska-Fidelus E. [Glioblastoma with BRAFV600E mutation and numerous metastatic foci: a case report]. Folia Neuropathol. 2019;57(1):72-79. Polish. doi: 10.5114/fn.2019.83833. IF: 1,278
- Peciak J, Stec WJ, Treda C, Ksiazkiewicz M, Janik K, **Popeda M**, Smolarz M, Rosiak K, Hulas-Bigoszewska K, Och W, Rieske P, Stoczynska-Fidelus E. Low Incidence along with Low mRNA Levels of EGFRvIII in Prostate and Colorectal Cancers Compared to Glioblastoma. J Cancer. 2017 Jan 1;8(1):146-151. doi: 10.7150/jca.16108. IF: 3,249
- Janik K, **Popeda M**, Peciak J, Rosiak K, Smolarz M, Treda C, Rieske P, Stoczynska-Fidelus E, Ksiazkiewicz M. Efficient and simple approach to in vitro culture of primary epithelial cancer cells. Biosci Rep. 2016 Dec 9;36(6):e00423. doi: 10.1042/BSR20160208. IF: 2,906
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Łączna wartość wskaźnika oddziaływania (IF):

- publikacje niewchodzące w skład rozprawy: 72,541
- wszystkie publikacje: 92,835

Łączna liczba cytowań: 116 (wg bazy Scopus, stan na dzień 09-06-2022)

Indeks Hirscha (H-index): 6 (wg bazy Scopus, stan na dzień 09-06-2022)

Przyznane projekty badawcze

- Charakterystyka molekularna i komórkowa przerzutów raka jelita grubego do płuc i wątroby – potencjalne implikacje kliniczne (ID: 2021/41/N/NZ4/03555); **PRELUDIUM 20**, **Narodowe Centrum Nauki**; 209 840 PLN; 2022-2025

- Profil i dynamika zmian poziomu cytokin w krwi obwodowej biorcy po zabiegu transplantacji serca – badanie pilotażowe (ID: 73-3324); **MŁODY TWÓRCA NAUKI**, Gdański Uniwersytet Medyczny – **Inicjatywa doskonałości – Uczelnia Badawcza**; 50 000 PLN; 2021-2022

Doniesienia konferencyjne – Autor prezentujący (łączna liczba: 7)

- **Popeda M**, Peksa R, Zok J, Bienkowski M, Biernat W. Divergent immune, stromal, and molecular patterns in lung and liver metastasis of colorectal cancer with potential clinical implications, EMBO | EMBL Symposium: Defining and defeating metastasis, 2022, Heidelberg, Niemcy – plakat
- **Popeda M**, Markiewicz A, Szade J, Welnicka-Jaskiewicz M, Bednarz-Knoll N, Zaczek AJ. Immune-related transcriptome landscape of luminal operable primary breast tumours – relation with CTCs presence and their EMT state. 5th Advances in Circulating Tumor Cells: Liquid Biopsy in its best, 2021, Kalamata, Grecja – plakat
- **Popeda M**, Stokowy T, Bednarz-Knoll N, Jurek A, Niemira M, Kretowski A, Kalinowski L, Szade J, Markiewicz A, Zaczek AJ. Upregulation of the NF-kappa B pathway-related genes in primary tumour is linked to the mesenchymal phenotype of circulating tumour cells in operable breast cancer. EACR-AACR-ASPIC 2020 Conference: Tumor Microenvironment, Lizbona, Portugalia – plakat
- **Popeda M**, Bednarz-Knoll N, Jurek A, Nagel A, Markiewicz A, Niemira M, Skokowski J, Kalinowski L, Zaczek AJ. Immune-related transcriptome profiles associated with negative prognostic factors for operable breast cancer. Molecular Biology and Immunology of Cancer – R&D perspectives: ScanBalt Forum 2019, Gdańsk, Poland – plakat
- **Popeda M**, Bednarz-Knoll N, Nagel A, Jurek A, Markiewicz A, Kryczka T, Szade J, Skokowski J, Zaczek AJ. Serum cytokine and chemokine profiles in primarily operable breast cancer patients. 5th International Conference of Cell Biology, 2019, Kraków, Poland – plakat
- **Popeda M**, Nagel A, Jurek A, Topa J, Markiewicz A, Seroczynska B, Skokowski J, Zaczek AJ. Clinical significance and biological role of plakoglobin expression in CTC-enriched blood fraction in early breast cancer patients. 14th YSA PhD Symposium, 2018, Wiedeń, Austria – plakat
- **Popeda M**, Janik K, Rosiak K, Smolarz M, Pawlowska R, Peciak J, Stec WJ, Ciechanowski M, Stoczynska-Fidelus E, Rieske P. Pharmacogenomic platform based on comprehensive molecular characterization as a promising tool to select novel anti-cancer polytherapies. XXIst Gliwice Scientific Meetings, 2017, Gliwice, Poland – plakat

Doniesienia konferencyjne – Współautor (łączna liczba: 23)

- **Popęda M**, Bieńkowski M, Topa J, Chakraborty P, Jolly MK, Szade J, Skokowski J, Wełnicka-Jaśkiewicz M, Żaczek AJ, Markiewicz A. EMT plasticity during luminal and triple-negative breast cancer dissemination with EMT-related changes in proliferation rate and stromal composition, EMBO | EMBL Symposium: Defining and defeating metastasis, 2022, Heidelberg, Niemcy – plakat
- Bienkowski M, **Popeda M**, Pillai M, Chakraborty P, Jolly MK, Myszczynski K, Artichowicz W, Jungnickel C, Markiewicz A. Bulk transcription-based EMT signatures suffer from stromal interference and mask the subtype-specific patterns of EMT program in breast cancer,

EMBO | EMBL Symposium: Defining and defeating metastasis, 2022, Heidelberg, Niemcy – plakat

- Senkus-Konefka E, **Popeda M**, Kunc M, Bienkowski M, Braun M, Lacko A, Radecka BS, Pikiel J, Litwiniuk M, Pogoda K, Izycka-Swieszewska E, Zaczek AJ, Biernat W. miRNA signatures of prognostic significance in single hormone receptor-positive breast cancer. 2022 ASCO Annual Meeting, Chicago, USA – doniesienie konferencyjne (streszczenie)
- Smentoch J, Wenta R, **Popeda M**, Muchlinska A, Niegowski P, Szade J, Zaczek A, Sowa M, Miszewski K, Matuszewski M, Bednarz-Knoll N. Phenotype and interactions of circulating tumor cells in tumor draining vein blood from d'Amico high risk prostate cancer patients: preliminary results, EACR Conference on Liquid Biopsies, 2022, Bergamo, Włochy – plakat
- Smentoch J, **Popeda M**, Wenta R, Matuszewski M, Sowa M, Szade J, Zaczek A, Semjonow A, Nastaly P, Bednarz-Knoll N. Clinical relevance and identification of molecular phenotype of keratins-negative prostate cancer. 5th Advances in Circulating Tumor Cells: Liquid Biopsy in its best, 2021, Kalamata, Grecja – plakat
- Pastuszak K, Łukasiewicz M, Żuk M, **Popęda M**, Łapińska-Szumczyk S, Łojkowska A, Best MG, In 't Veld SGJG, Würdinger T, Stokowy T, Żaczek A, Jassem JJ, Supernat A. Tumor-Educated Platelets in gynecological cancers. EACR Liquid Biopsies Virtual Event 2020 – plakat
- Kunc M, **Popeda M**, Szalkowska A, Niemira M, Lacko A, Radecka BS, Braun M, Pikiel J, Litwiniuk M, Pogoda K, Szwajkosz A, Izycka-Swieszewska E, Zaczek AJ, Biernat W, Senkus-Konefka E. microRNA expression profiles of single hormone receptor-positive breast cancers. ESMO Breast Cancer Virtual Meeting 2020 – plakat
- Nagel A, **Popeda M**, Jurek A, Skokowski J, Zielinski J, Bednarz-Knoll N, Markiewicz A, Zaczek AJ. Cancer associated fibroblasts subtypes play a dual role in different molecular subtypes of breast cancer. EACR-AACR-ASPIC 2020 Conference: Tumor Microenvironment, Lizbona, Portugalia – plakat
- Jurek A, **Popeda M**, Bednarz-Knoll N, Szade J, Skokowski J, Zaczek AJ. Prognostic significance and molecular profile of tumor associated macrophages in breast cancer. Molecular Biology and Immunology of Cancer – R&D perspectives: ScanBalt Forum 2019, Gdańsk, Poland – plakat
- Nagel A, Jurek A, **Popeda M**, Szade J, Skokowski J, Zielinski J, Bednarz-Knoll N, Zaczek AJ. Cancer associated fibroblasts subpopulations defined by ERα36 expression and their action on different breast cancer subtypes. Molecular Biology and Immunology of Cancer – R&D perspectives: ScanBalt Forum 2019, Gdańsk, Poland – plakat
- Kowalski J, **Popeda M**, Karczewska J, Wrona A, Dziadziuszko R, Biernat W, Marek-Trzonkowska N, Hupp T. Immunohistochemical study of tumour-infiltrating lymphocytes in non-small cell lung cancer. Molecular Biology and Immunology of Cancer – R&D perspectives: ScanBalt Forum 2019, Gdańsk, Poland – plakat
- Jurek A, **Popeda M**, Bednarz-Knoll N, Szade J, Skokowski J, Zaczek AJ. Correlation of tumor associated macrophages infiltration into tumor stroma with cancer progression in breast cancer. 5th International Conference of Cell Biology, 2019, Kraków, Poland – plakat
- Nagel A, Jurek A, **Popeda M**, Szade J, Skokowski J, Zielinski J, Zaczek AJ. Role of cancer associated fibroblasts in different breast cancer subtypes. 5th International Conference of Cell Biology, 2019, Kraków, Poland – plakat
- Bednarz-Knoll N, Kryczka T, Szade J, Markiewicz A, **Popęda M**, Grieb P, Seroczyńska B, Skokowski J, Żaczek AJ. Preoperative high platelets counts and low hemoglobin correlate to mesenchymal phenotype of circulating tumor cells and worse clinical outcome in human

breast carcinoma. The 3rd Congress of Polish Biosciences "BIO 2018 – Through interdisciplinary approach into new solutions", 2018, Gdańsk, Poland – prezentacja

- Jurek A, Nagel A, **Popeda M**, Topa J, Zaczek AJ. Influence of tumor-associated macrophages on breast cancer aggressiveness. 14th YSA PhD Symposium, 2018, Wiedeń, Austria – plakat
- Topa J, Markiewicz A, Nagel A, Szade J, Majewska H, Skokowski J, Seroczynska B, Stokowy T, Welnicka-Jaskiewicz M, **Popeda M**, Jurek A, Zaczek AJ. The analysis of cadherin switch and epithelial-mesenchymal transition activation in circulating tumor cells from breast cancer patients. 14th YSA PhD Symposium, 2018, Wiedeń, Austria – plakat
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- Peciak J, Janik K, **Popeda M**, Stec WJ, Treda C, Rosiak K, Stoczynska-Fidelus E, Rieske P. Detecting the hardly detectable: the best approach to analyse Epidermal Growth Factor Receptor viariant III (EGFRvIII). XXIst Gliwice Scientific Meetings, 2017, Gliwice, Poland – plakat – wyróżnienie
- Wlodarczyk A, Grot D, **Popeda M**, Janik K, Wasiak K, Ciechanowski M, Rieske P. IL-2 signal peptide is not sufficient to efficiently purify recombinant K-RAS protein from culture medium. XXIst Gliwice Scientific Meetings, 2017, Gliwice, Poland – plakat
- Płuciennik E, Nowakowska M, **Popęda M**, Gałdyszyńska M, Kośla K, Pospiech K, Baryła I, Styczeń-Binkowska E, Orzechowska M, Jędroszka D, Bednarek AK. The role of WWOX gene in biology of endometrial cells. XXth Gliwice Scientific Meetings, 2016, Gliwice, Poland – plakat
- Janik K, **Popeda M**, Rosiak K, Smolarz M. Stosowanie politerapii celowanych w oparciu o profil molekularny komórek nowotworowych na przykładzie linii glejaka wielopostaciowego (DK-MG). II Konferencja Biotechnologiczna BioBetter, 2016, Łódź, Polska – plakat
- Rieske P, Stoczyńska-Fidelus E, Piaskowski S, Janik K, Smolarz M, **Popęda M**, Rosiak K. Rational combinations of targeted anticancer therapies. 42nd Congress of the International Society of Oncology and Biomarkers, 2015 October 3-7, Zakopane, Poland – prezentacja
- Płuciennik E, Nowakowska M, **Popęda M**, Pospiech K, Kośla K, Baryła I, Styczeń-Bińkowska E, Bednarek AK. The influence of WWOX gene on invasiveness of estrogen independent endometrial cancer cell line. 19th World Congress on Advances in Oncology and 17th International Symposium on Molecular Medicine, 2014, Ateny, Grecja – plakat

Nagrody i wyróżnienia

- Nagroda zespołowa Rektora Gdańskiego Uniwersytetu Medycznego II stopnia za badania nad profilowaniem krążących komórek nowotworowych u chorych na raka piersi – 2020
- Nagroda zespołowa Rektora Uniwersytetu Medycznego w Łodzi I stopnia za cykl publikacji na temat „Określenie mechanizmów regulacji ekspresji genu WWOX oraz jego udziału w przejściu epitelialno-mezenchymalnym w kancerogenezie” – 2015
- Zwiększone stypendium z dotacji projakościowej dla najlepszych doktorantów MWB UG-GUMed – 2017/2018, 2020/2021, 2022/2021
- Stypendium Rektora Uniwersytetu Gdańskiego dla najlepszych doktorantów MWB UG-GUMed – 2020/2021, 2021/2022