Gdański Uniwersytet Medyczny

Wydział Lekarski

Rozprawa doktorska

Analiza kliniczno-patologiczna i ocena profilu ekspresji mikroRNA raków piersi wykazujących ekspresję jednego receptora hormonalnego (ER+/PgR– i ER–/PgR+)

A clinicopathological analysis and microRNA profiling of single hormone receptor-positive breast cancer (ER+/PgR– and ER–/PgR+)

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SPIS TREŚCI

WYKAZ PRAC WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ / LIST OF MANUSCRIPTS INCLUDED IN THE DOCTORAL DISSERTATION

Kunc M, Biernat W, Senkus-Konefka E.

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IF 8,332 | MNiSW: 40 pkt | praca poglądowa

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IF 6.639| MNiSW: 140 pkt | praca poglądowa

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. 2020; 20;10(9):617. doi: 10.3390/diagnostics10090617.

IF 3,706 | MNiSW: 70 pkt | praca oryginalna

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High expression of progesterone receptor may be an adverse prognostic factor in oestrogen receptor-negative/progesterone receptor-positive breast cancer: results of comprehensive re-evaluation of multi-institutional case series.

Pathology. 2022; 21;S0031-3025(21)00542-0. doi: 10.1016/j.pathol.2021.10.003.; w druku (in press)

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Rak piersi, receptor estrogenowy, receptor progesteronowy, immunohistochemia, mikroRNA, rokowanie, analiza przeżycia

Breast cancer, estrogen receptor, progesterone receptor, immunohistochemistry, microRNA, prognosis, survival analysis

STRESZCZENIE W JĘZYKU POLSKIM

I. WYKAZ STOSOWANYCH SKRÓTÓW

ASCO/CAP – American Society of Clinical Oncology/College of American Pathologists

CI – przedział ufności

DFS – czas przeżycia wolnego od choroby

ER – receptor estrogenowy

FFPE – utrwalony w formalinie, zatopiony w parafinie

HER2 – receptor dla naskórkopochodnego czynnika wzrostu typu 2

HR – względny hazard

miRNA – mikroRNA

OS – całkowity czas przeżycia

PgR – receptor progesteronowy

SHRP – raki z ekspresją jednego receptora hormonalnego

TCGA – The Cancer Genome Atlas

II. WPROWADZENIE

Rak piersi jest najczęściej występującym nowotworem złośliwym oraz drugą, po raku płuca, najczęstszą przyczyną zgonu z powodu choroby nowotworowej u kobiet w Polsce [1]. Ocena rokowania oraz wybór terapii w raku piersi zależą od wielu czynników, szczególnie od stopnia zaawansowania i ekspresji biomarkerów immunohistochemicznych: receptora estrogenowego α (ER), receptora progesteronowego (PgR), receptora dla naskórkopochodnego czynnika wzrostu typu 2 (HER2) oraz indeksu proliferacyjnego Ki67 [2]. Raki piersi niewykazujące ekspresji receptorów steroidowych charakteryzują się bardziej agresywnym przebiegiem klinicznym i opornością na hormonoterapię. W przeciwieństwie do nich raki o fenotypie ER-pozytywny (+) i PgR-pozytywny (+) mają łagodniejszy przebieg kliniczny i większą wrażliwość na terapię hormonalną.

Wyjątkowo interesującą grupę raków piersi stanowią te, w których stwierdza się ekspresję tylko jednego typu receptora hormonalnego (tj. ER albo PgR), tzw. *single hormone receptor-positive breast cancers* (SHRP) [3].

Pierwotny brak lub utrata ekspresji PgR może być wykładnikiem potencjalnej oporności na hormonoterapię. Guzy o fenotypie ER(+)/PgR-negatywny (–) stanowią około 12-24% wszystkich raków piersi [4]. Natomiast częstość występowania raków piersi o fenotypie ER-negatywny (–)/PgR(+) w latach 90-tych XX wieku wynosiła około 10-15%. Nowsze dane wskazują jednak, że są one o wiele rzadsze i stanowią około 0,5-1,5% raków piersi [5]. Zmniejszenie rozpoznawalności tego fenotypu wiąże się prawdopodobnie z zastąpieniem metod radioimmunologicznych nowoczesną immunohistochemiczną oceną ekspresji receptorów steroidowych.

W przeszłości wielu autorów podważało istnienie raków ER(–)/PgR(+). Kontrowersje wokół tego fenotypu są wieloczynnikowe. Głównym z nich jest obserwacja, że w komórkach raka piersi ekspresja PgR jest indukowana głównie przez ER [6]. Wynika z tego, że w rakach piersi o fenotypie ER(–) ekspresja PgR nie powinna być obserwowana. Część badaczy sugeruje więc, że fenotyp ER(–)/PgR(+) jest technicznym artefaktem [7], ale nawet przy zastosowaniu bardzo restrykcyjnych procedur raki ER(–)/PgR(+) nadal są rozpoznawane [8]. Raki SHRP charakteryzuje szereg podobieństw. W porównaniu do raków ER(+)/PgR(+) mają

agresywniejszy przebieg kliniczny, częściej są oporne na hormonoterapię i wykazują większą wrażliwość na chemioterapię [9]. Część danych wskazuje jednak na istotne różnice, szczególnie w epidemiologii: raki ER(+)/PgR(–) występują częściej u pacjentek powyżej 60. roku życia, natomiast raki ER(–)/PgR(+) zwykle rozpoznawane są u młodszych pacjentek [10,11].

III. CELE PRACY

1. Podsumowanie obecnego stanu wiedzy dotyczącej raków piersi o immunofenotypie ER(–)/PgR(+) z uwzględnieniem ich epidemiologii, etiologii, patogenezy i przebiegu klinicznego
2. Podsumowanie obecnego stanu wiedzy dotyczącej raków piersi o immunofenotypie ER(+)/PgR(–) z naciskiem na ich cechy molekularne i związane z tym implikacje kliniczno-terapeutyczne
3. Określenie profilu ekspresj mikroRNAi (miRNA) w rakach piersi SHRP w materiale własnym oraz ze współpracujących ośrodków i zwalidowanie wyników przy użyciu danych z bazy *The Cancer Genome Atlas* (TCGA) [12].
4. Reewaluacja rozpoznań raków piersi pierwotnie rozpoznanych jako ER(–)/PgR(+) w materiale własnym oraz ze współpracujących ośrodków przy użyciu immunohistochemii. Analiza cech kliniczno-patologicznych w grupie potwierdzonych przypadków ER(–)/PgR(+) w porównaniu do przypadków ze zmienionym rozpoznaniem.

IV. MATERIAŁ I METODY

1. Przegląd literatury

a) Raki piersi ER(–)/PgR(+)

Przeprowadzono wyszukiwanie w bazie PubMed/MEDLINE używając następujących słów kluczowych: „breast cancer", „estrogen receptor", „progesterone receptor". Analizę ograniczono do lat 1990-2017. Istotne artykuły zostały wybrane przez doktoranta. Ponadto, dokonano przeglądu piśmiennictwa wybranych artykułów. Ostateczny zakres przeglądu literatury został zaakceptowany przez panel współautorów.

b) Raki piersi ER(+)/PgR(–)

Przeprowadzono wyszukiwanie w bazie MEDLINE używając następujących słów kluczowych: „breast cancer", „estrogen receptor-positive", „progesterone receptor-negative". Analizę ograniczono do lat 2006-2021. Istotne artykuły zostały wybrane przez doktoranta. Ponadto, dokonano przeglądu piśmiennictwa wybranych artykułów. Ostateczny zakres przeglądu literatury został zaakceptowany przez panel współautorów. Ryciny zostały przygotowane z użyciem platformy *Biorender.com*.

2. Ocena ekspresji miRNA w rakach SHRP

W badaniu wykorzystano 36 przypadków raków piersi wykazujących ekspresję jednego receptora hormonalnego [18 ER(+)/PgR(–) i 18 ER(–)/PgR(+)] dobranych pod kątem zbliżonych cech kliniczno-patologicznych (wiek, stopień zróżnicowania, status HER2, indeks Ki67). Całkowity RNA wyizolowano z archiwalnych bloków parafinowych utrwalonych w formalinie (ang. *formalin-fixed paraffin-embedded tissue blocks,* FFPE) przy użyciu RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Invitrogen, Carlsbad, CA, USA). Stężenie i czystość RNA zostały określone przy zastosowaniu spektrofotometru NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA).

Wyizolowany RNA został poddany profilowaniu miRNA z użyciem nCounter Human v3 miRNA Expression Assay (NanoString Technologies, Seattle, WA, USA) w Centrum Badań Klinicznych w Białymstoku (dr Magdalena Niemira i dr Anna

Szałkowska). Normalizacja i wstępna analiza surowych danych została przeprowadzona z użyciem oprogramowania nSolver 4.0. Ponadto, celem walidacji uzyskanych wyników, przeanalizowano dane uzyskane z bazy TCGA z wybranych 67 przypadków raków piersi SHRP [12]. Następnie zidentyfikowano miRNA ulegające zróżnicowanej ekspresji w rakach ER(–)/PgR(+) oraz ER(+)/PgR(–) w zależności od ich statusu HER2.

Celem zidentyfikowania targetowych mRNA dla zidentyfikowanych miRNA wykorzystano bazę miRNET 2.0 oraz przeprowadzono analizę funkcjonalną (Gene Ontology Biological Processes, GO BP) z użyciem Functional Annotation Tool (DAVID Bioinformatics Resources 6.81) [13–15].

3. Reewaluacja rozpoznań raków piersi ER(–)/PgR(+)

Do badania zakwalifikowano 135 przypadków raków piersi z pierwotnie określonym fenotypem ER(–)/PgR(+) i zebrano bloki FFPE zawierające tkanki nowotworowe uzyskane z 86 biopsji gruboigłowych lub biopsji wspomaganych próżnią i z 76 zabiegów operacyjnych. W 27 przypadkach dostępny był zarówno materiał z biopsji, jak i pooperacyjny. Następnie preparaty wybarwiono z użyciem trzech klonów przeciwciał anty-ER (1D5, Dako; EP1, Dako; SP1, Roche) oraz jednego klonu przeciwciała anty-PgR (636, Dako) zgodnie z instrukcjami producentów. W kolejnym kroku dokonano oceny uzyskanych barwień. W przypadkach ER(–)/PgR(–) dokonano dodatkowo barwienia drugim klonem przeciwciała anty-PgR (1E2, Roche).

Ocenę ekspresji receptorów steroidowych przeprowadzono według wytycznych American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) i odczyn jądrowy w ≥1% komórek raka był uznawany za pozytywny [16].

4. Metody statystyczne

Analiza statystyczna została przeprowadzona w środowisku statystycznym R oraz przy użyciu oprogramowania Statistica 13 (Tibco, CA, USA) [17]. Zmienne kategoryczne były porównywane testem dokładnym Fishera lub testem chi-kwadrat z korekcją Yatesa. Rozkład normalny oceniono przy pomocy testu Shapiro-Wilka. Zmienne ilościowe były analizowane z użyciem testów U Manna-Whitney'a, Kruskala-Wallisa, testu t-Studenta oraz dwustronnej analizy wariancji ANOVA. Korelacje

pomiędzy wartościami liniowymi były analizowane z użyciem testów Spearmana i Pearsona.

Krzywe Kaplana-Meiera zostały użyte do oszacowania wskaźników przeżycia. Wartości względnego hazardu (hazard ratio, HR) z przedziałami ufności (confidence intervals, CIs) zostały wyliczone z użyciem regresji Coxa.

Wartości $p \leq 0{,}05$ były uznawane za statystycznie istotne. W przypadku porównań wielokrotnych używano poprawki Benjaminiego-Hochberga.

V. OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

W skład mojej pracy doktorskiej wchodzą cztery artykuły (dwa przeglądowe i dwa oryginalne) opublikowane w międzynarodowych czasopismach indeksowanych w Liście Filadelfijskiej. Publikacje dotyczą problematyki raków piersi SHRP – ER(–)/PgR(+) oraz ER(+)/PgR(–) ze szczególnym uwzględnieniem ich patogenezy, cech molekularnych i charakterystyki kliniczno-patologicznej.

Publikacja 1.

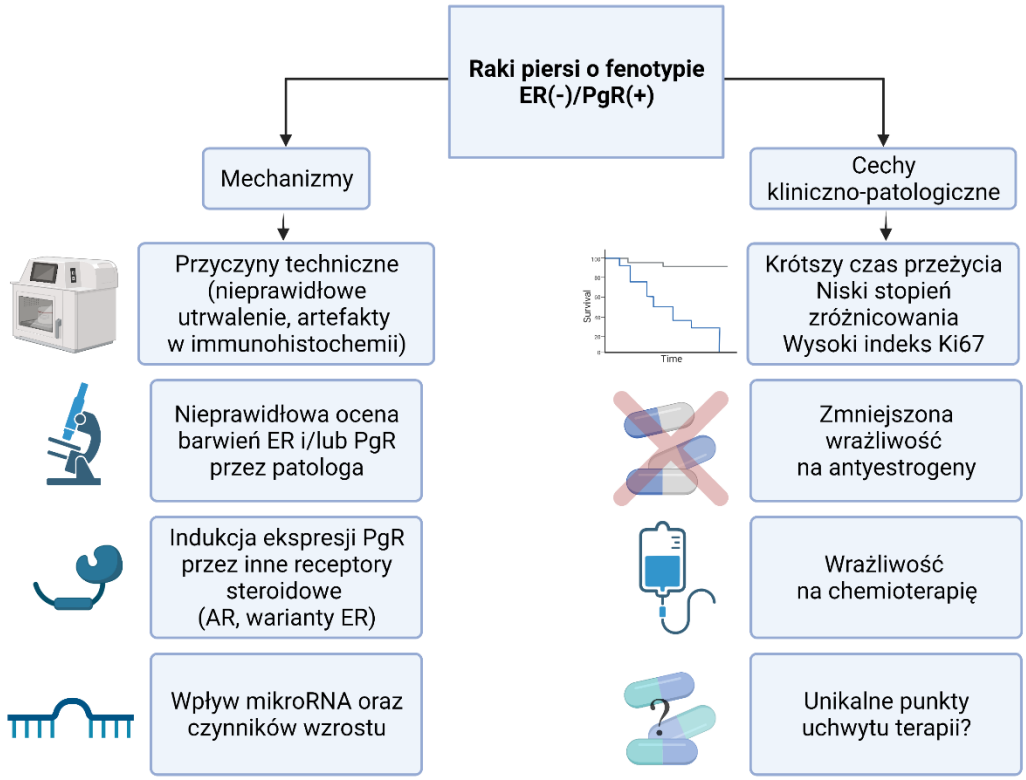
Kunc M, Biernat W, Senkus-Konefka E.: *Estrogen receptor-negative progesterone receptor-positive breast cancer - "Nobody's land" or just an artifact?* Cancer Treatment Reviews. 2018; 67:78-87.

Nowotwory o fenotypie ER(–)/PgR(+) stanowią najmniej liczną, ale jednocześnie bardzo kontrowersyjną, grupę raków piersi. Z powodu rzadkiego występowania tego fenotypu niewiele wiadomo na temat ich etiopatogenezy, profilu molekularnego oraz cech kliniczno-patologicznych. W naszym artykule przeprowadziliśmy szczegółowy przegląd piśmiennictwa celem podsumowania obecnego stanu wiedzy na temat tych nowotworów.

W pierwszej części publikacji omówiliśmy wiodące hipotezy próbujące wyjaśnić mechanizm powstawania raków piersi o fenotypie ER(–)/PgR(+), uwzględniając błędy przedlaboratoryjne i laboratoryjne, heterogenność komórkową guza, stosowanie różnych kryteriów oceny uzyskanych odczynów immunohistochemicznych, wpływ innych receptorów, czynników wzrostu oraz miRNA (Rycina 1.). Omówiliśmy również cechy molekularne raków ER(–)/PgR(+).

Następnie przedstawiliśmy epidemiologię i cechy kliniczno-patologiczne raków piersi ER(–)/PgR(+) (Rycina 1.). Niektóre dane wskazują, że raki piersi ER(–)/PgR(+) występują u kobiet młodszych i względnie częściej u Afroamerykanek w porównaniu do raków ER(+)/PgR(+). Ponadto, często charakteryzują się niskim stopniem zróżnicowania histologicznego, wysokim indeksem proliferacyjnym Ki67, a molekularnie dominują wśród nich guzy klasyfikowane jako typ bazalny.

Większość danych wskazuje, że pacjentki z fenotypem ER(–)/PgR(+) mają pośrednie rokowanie wyrażone całkowitym czasem przeżycia (OS) oraz czasem przeżycia wolnego od choroby (DFS) między rakami podwójnie pozytywnymi i negatywnymi. Ponadto, wykazują one względnie niską wrażliwość na hormonoterapię i względnie wysoką wrażliwość na chemioterapię. Planowanie leczenia systemowego może być ułatwione dzięki określeniu sygnatur molekularnych, np. PAM50.

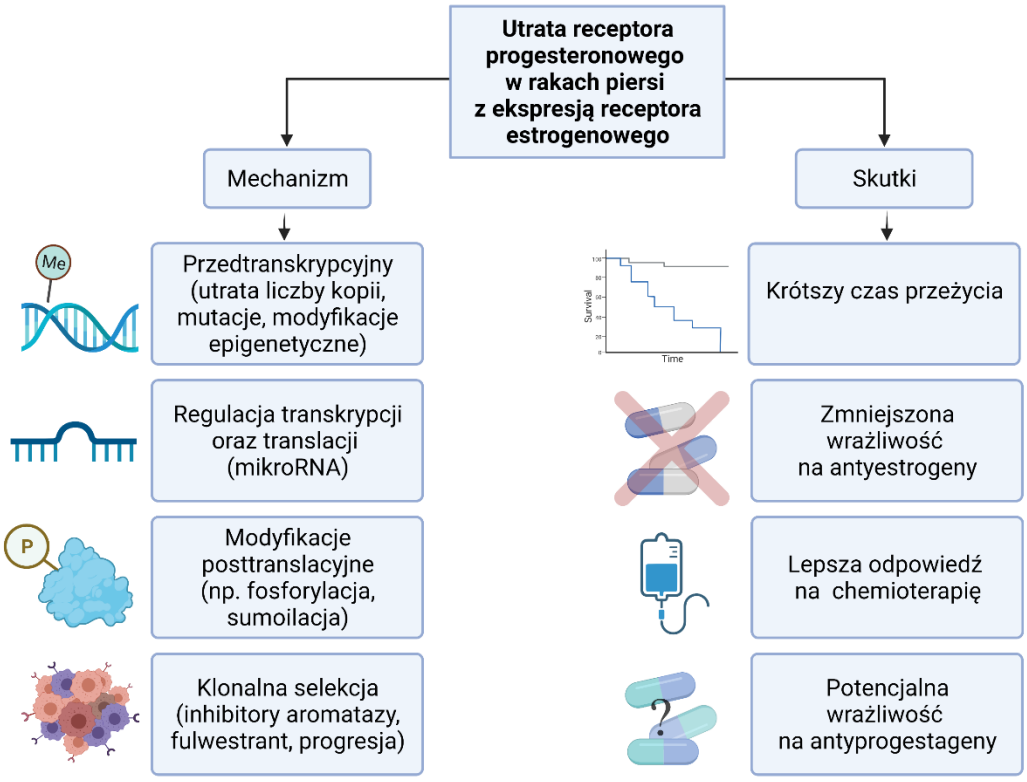


Rycina 1. Podsumowanie potencjalnych przyczyn rozpoznawania oraz cech kliniczno-patologicznych raków piersi o fenotypie ER(–)/PgR(+).

Publikacja 2.

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. 2021; 23;13(19):4755.

Charakterystyka kliniczno-patologiczna raków piersi ER(–)/PgR(+) jest dość dobrze poznana, ale patogeneza i cechy molekularne pozostają obiektem intensywnych badań. W naszej publikacji skupiliśmy się na postępach w zrozumieniu mechanizmów i biologicznych skutków utraty ekspresji PgR w rakach ER(+) (Rycina 2.).



Rycina 2. Podsumowanie mechanizmów utraty ekspresji PgR w rakach piersi ER(+) i związanych z tym skutków kliniczno-patologicznych.

W pierwszej części omawiamy mechanizmy utraty ekspresji PgR w raku piersi. Wśród proponowanych mechanizmów wymienia się utratę na poziomie genetycznym (np. utrata liczby kopii genu, mutacje, modyfikacje epigenetyczne), zmiany regulacji transkrypcji (miRNA) lub modyfikacji post-translacyjnych (fosforylacja, metylacja, sumoilacja). Brak ekspresji PgR może dotyczyć pierwotnego nowotworu lub być skutkiem klonalnej selekcji. Mechanizm utraty ekspresji PgR może mieć ogromne znaczenie biologiczne. Część raków piersi o bardzo wysokiej aktywności transkrypcyjnej PgR może być PgR(–) w immunohistochemii z powodu szybkiej degradacji receptora w proteasomach. Takie zjawisko szczególnie często występuje w przypadku raków, w których aktywowane są receptory dla czynników wzrostu, np. HER2. Zwracamy uwagę, że takie raki mogą być paradoksalnie potencjalnie wrażliwe na terapię antyprogestagenami.

W kolejnej części skupiamy się na utracie PgR pod wpływem leczenia lub w przypadku wznowy nowotworu. Utrata ekspresji PgR w rakach ER(+) może wskazywać

na klonalną selekcję komórek, które są oporne na hormonoterapię lub chemioterapię i jest związana z gorszym rokowaniem.

Ponadto, omówiliśmy profil genetyczny i biologię raków piersi z utratą ekspresji PgR. W kompleksowy sposób opisujemy interakcje pomiędzy ER i PgR w komórkach ER(+)/PgR(+) oraz konsekwencje utraty PgR, w szczególności zwracając uwagę na potencjalną rolę innych receptorów.

Publikacja 3.

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. 2020; 20;10(9):617.

Cząsteczki miRNA są krótkimi, niekodującymi oligonukleotydami zaangażowanymi w proces regulacji transkrypcji i translacji. Mogą one pełnić rożne role w procesie nowotworzenia i działać jako cząsteczki supresorowe (tzw. tsmiRs) lub onkogeny (tzw. oncomiRs). W rakach piersi niektóre sygnatury miRNA mają znaczenie prognostyczne, predykcyjne i diagnostyczne. W naszym badaniu skupiliśmy się na przeanalizowaniu profilu ekspresji miRNA w rakach piersi SHRP, tj. ER(+)/PgR(–) oraz ER(–)/PgR(+). Według naszej wiedzy jest to pierwsze badanie tego typu na świecie.

Do badania włączyliśmy po 18 przypadków raków ER(+)/PgR(–) i ER(–)/PgR(+) z dostępnym materiałem tkankowym w postaci FFPE i ze znanym statusem HER2. Z powodu niskiej jakości próbek, cztery z nich zostały wyeliminowane z dalszych analiz, pozostawiając 14 przypadków ER(+)/PgR(–) i 18 przypadków ER(–)/PgR(+). Po wprowadzeniu poprawki na porównania wielokrotne nie znaleźliśmy sygnatury miRNA różnicującej raki ER(–)/PgR(+) i ER(+)/PgR(–). Jednakże, w przypadku 8 miRNA zaobserwowaliśmy statystyczny trend w kierunku zróżnicowanej ekspresji między grupami: raki ER(+)/PgR(–) wykazywały wyższą ekspresję miRNA o znanym związku z pozytywnym statusem ER (miR-30a-5p, miR-29c-3p, miR-141-3p), a raki ER(–)/PgR(+) wykazywały wyższą ekspresję miRNA o znanym związku z rakami podwójnie i potrójnie negatywnymi (miR-92a-3p, miR-424-5p). W przypadku jednego z tych miRNA (miR-29c-3p) związek został potwierdzony w kohorcie TCGA

(p=0,024; test t-Studenta). Status HER2 w naszej kohorcie był związany z 33 miRNA. W przypadku 4 miRNA (miR-1180-3p, miR-223-3p, miR-30d-5p, miR-195-5p) związek ze statusem HER2 został potwierdzony w bazie TCGA.

Publikacja 4.

Kunc M, Pęksa R, Cserni G, Iżycka-Świeszewska E, Łacko A, Radecka B, Braun M, Pikiel J, Litwiniuk M, Pogoda K, Szwajkosz A, Biernat W, Senkus E. High expression of progesterone receptor may be an adverse prognostic factor in oestrogen receptor-negative/progesterone receptor-positive breast cancer: results of comprehensive re-evaluation of multi-institutional case series. Pathology. 2022; 21;S0031-3025(21)00542-0; w druku (in press)

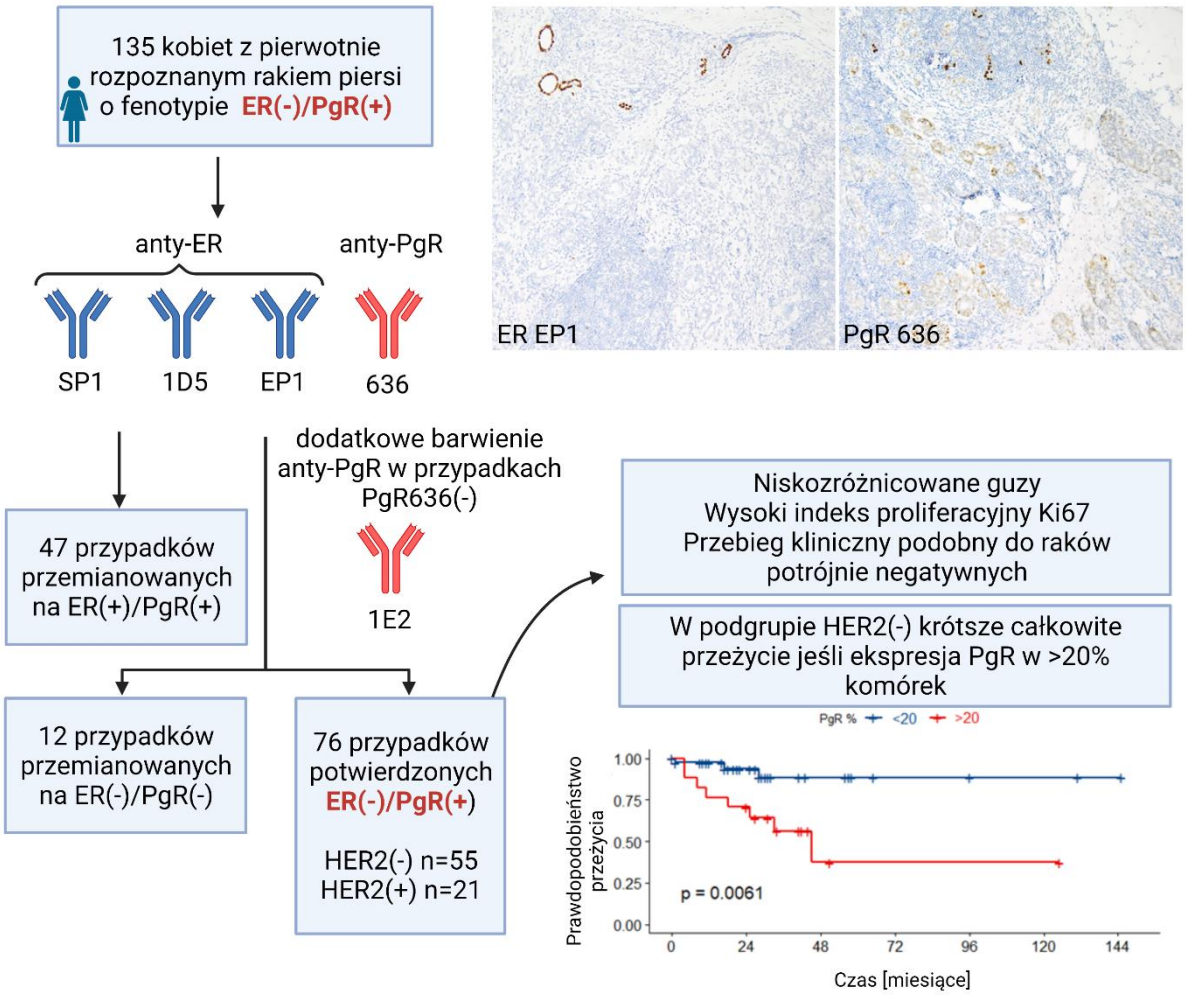
Celem tego badania była walidacja rozpoznań raków piersi o fenotypie ER(–)/PgR(+) zebranych z kilku polskich i węgierskich ośrodków. Do badania retrospektywnie zakwalifikowaliśmy 135 przypadków. Przeprowadziliśmy barwienia immunohistochemiczne z użyciem trzech klonów przeciwciał anty-ER i dwóch klonów przeciwciał anty-PgR.

Ogólna zgodność pomiędzy trzema klonami anty-ER była dobra (kappa Fleissa 0,76), ale w 21 przypadkach (15,5%) stwierdzono brak zgodności pomiędzy klonami. Największe niezgodności zaobserwowaliśmy między klonami SP1 i EP1. Znaczące różnice zanotowaliśmy także w przypadku przeciwciał anty-PgR. Spośród 42 przypadków PgR(–) w reakcji z klonem 636, 32 (76,2%) okazały się być PgR(+) w barwieniu z użyciem klonu 1E2.

Finalnie, potwierdziliśmy fenotyp ER(–)/PgR(+) w 76 przypadkach (56,3%). W 47 przypadkach (34,8%) zmieniliśmy rozpoznanie na ER(+)/PgR(+), a w 12 (8,9%) na ER(–)/PgR(–) (Rycina 3.). Potwierdzone przypadki ER(–)/PgR(+) charakteryzowały się w większości niskim stopniem zróżnicowania (grade 3) i wysokim indeksem proliferacyjnym Ki67.

W grupie raków HER2(–) najdłuższe OS stwierdziliśmy w grupie z rozpoznaniem zmienionym na ER(+)/PgR(+). Potwierdzone przypadki ER(–)/PgR(+), raki potrójnie negatywne oraz raki ER(+) z rozbieżnymi wynikami barwień między klonami, charakteryzowały się krótszym OS. Następnie zbadaliśmy, czy poziom ekspresji PgR wpływał na rokowanie w grupie ER(–)/PgR(+)/HER2(–). Co zaskakujące, stwierdziliśmy,

że grupa z ekspresją PgR w >20% komórek raka, miała znacząco krótszy OS od grupy z ekspresją PgR obecną w <20% komórek nowotworu. Wpływ PgR utrzymał się w analizie wieloczynnikowej kontrolowanej stopniem zaawansowania (HR 5,0, 95% CI 1,3-19,2, p=0,019).



Rycina 3. Przebieg rewaluacji rozpoznań raków piersi ER(–)/PgR(+). Zdjęcia mikroskopowe przedstawiają reprezentatywny potwierdzony przypadek ER(–)/PgR(+) z wewnętrzną kontrolą pozytywną.

VI. PODSUMOWANIE

Ocena ekspresji receptorów steroidowych za pomocą immunohistochemii pozwala na określenie rokowania i zaplanowanie leczenia u pacjentek chorych na raka piersi. W mojej pracy doktorskiej podsumowałem obecny stan wiedzy dotyczący raków piersi o fenotypie ER(–)/PgR(+) oraz ER(+)/PgR(–). Raki piersi SHRP posiadają wiele odrębnych cech w porównaniu do raków podwójnie pozytywnych i podwójnie negatywnych. Ich unikalny fenotyp sprawia, że mogą być oporne na hormonoterapię, ale jednocześnie wykazywać zwiększoną wrażliwość na chemioterapię.

Ponadto, przeanalizowaliśmy profile ekspresji miRNA raków piersi SHRP. Wykazaliśmy, że sygnatura miRNA tych nowotworów jest przede wszystkim zależna od statusu HER2. Niemniej jednak, zaobserwowaliśmy związek kilku miRNA ze statusem ER i PgR. Nasze badania stanowią punkt wyjścia do kolejnych badań translacyjnych nad związekim miRNA z ekspresją ER i PgR w raku piersi.

Wykazaliśmy, że raki piersi o fenotypie ER(–)/PgR(+) stanowią rzeczywistą i unikalną grupę nowotworów. Charakteryzują się one niskim stopniem histologicznego zróżnicowania oraz wysokim indeksem proliferacyjnym Ki67. Co więcej, wykazaliśmy, że w grupie raków ER(–)/PgR(+)/HER2(–) ekspresja PgR w >20% komórek raka wiąże się z krótszym OS. Podważa to istniejący paradygmat, według którego wyższa ekspresja receptorów steroidowych w raku piersi koreluje z lepszym rokowaniem.

SUMMARY IN ENGLISH

I. LIST OF ABBREVIATIONS

ASCO/CAP – American Society of Clinical Oncology/College of American Pathologists

CI – confidence interval

DFS – disease-free survival

ER – estrogen receptor

FFPE – formalin-fixed paraffin-embedded

HER2 – human epidermal growth factor receptor 2

HR – hazard ratio

miRNA – microRNA

OS – overall survival

PgR – progesterone receptor

SHRP – single hormone receptor-positive breast cancers

TCGA – The Cancer Genome Atlas

II. INTRODUCTION

Breast cancer is the most common malignancy and the second most common cause of cancer-related death in Polish females [1]. The assessment of prognosis and the selection of treatment is dependent on multiple factors, especially on the stage and the expression of immunohistochemical biomarkers: estrogen receptor α (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), and proliferation index Ki67 [2]. Steroid hormone receptor-negative breast cancers are usually characterized by an aggressive clinical course and resistance to endocrine therapy. On the other hand, ER positive (+) and PgR-positive (+) breast cancer are usually sensitive to endocrine treatment and have a less aggressive clinical course.

Single hormone receptor-positive (SHRP) breast cancers [i.e. either ER(+) or PgR(+)] constitute an especially interesting category of breast malignancies [3]. ER(+)/PgR-negative (–) tumors constitute approximately 12-24% of all breast cancers [4]. Primary lack of PgR expression or its loss during treatment of relapse may reflect the resistance to endocrine therapy. On the other hand, the described frequency of ER-negative (–)/PgR(+) breast cancer in the 90s of the twentieth century was about 10-15%, but more recent data indicate that they consist only of 0.5-1.5% of all breast cancers [5]. The decline in the frequency of their diagnosis is most likely a consequence of the shift from radioimmunological assays to modern methods of hormone receptors assessment like immunohistochemistry. Some authors postulate that ER(–)/PgR(+) breast cancers do not exist. The controversies around this phenotype are associated with a few factors. First of all, in breast cancer cells the expression of PgR is induced by ER [6]. Hence, some experts suggest that ER(–)/PgR(+) phenotype is a technical artifact [7]. Nevertheless, even when using restrictive procedures ER(–)/PgR(+) breast cancers are still identified [8]. SHRP breast cancers share several features. They are characterized by a more aggressive clinical behavior, more frequent resistance to endocrine therapy, and higher sensitivity to chemotherapy when compared to double-positive breast cancers [9]. However, some differences also exist, especially in epidemiological characteristics: ER(+)/PgR(–) breast cancers are more common in patients >60 years old, whereas ER(–)/PgR(+) tumors tend to occur in younger patients [10,11].

III. AIMS OF THE STUDY

1. To characterize and describe the current state of knowledge on ER(–)/PgR(+) breast cancer, with a special focus on their epidemiology, etiology, pathogenesis, and clinical course.
2. To characterize and describe the current state of knowledge on ER(+)/PgR(–) breast cancer with a special focus on their molecular features and associated clinicopathological implications.
3. To perform microRNA (miRNA) profiling in the group of SHRP breast cancer in our material and from cooperating centers and to validate the findings with the use of The Cancer Genome Atlas (TCGA) data.
4. To reevaluate ER(–)/PgR(+) diagnoses in our material and from cooperating centers and to describe the clinicopathological features of confirmed ER(–)/PgR(+) cases compared to recategorized cases.

IV. MATERIALS AND METHODS

1. Literature review

a) ER(–)/PgR(+) breast cancer

A PubMed/MEDLINE search was performed to identify all original and review articles addressing ER(−)/PgR(+) breast cancer. The following keywords were included: “breast cancer”, “estrogen receptor”, “progesterone receptor”. The analysis was restricted to the period between 1990 and 2017. Relevant papers were selected by the Ph.D. candidate with the agreement of the expert panel of coauthors. Bibliographies and related articles were also reviewed.

b) ER(+)/PgR(–) breast cancer

A MEDLINE search was performed with the use of the following keywords: “breast cancer”, “estrogen receptor-positive”, “progesterone receptor-negative”. The analysis was restricted to the period between 2006 and 2021. Relevant papers were selected by the Ph.D. candidate with the agreement of the expert panel of coauthors. Bibliographies and related articles were also reviewed. The figures were created with the Biorender.com platform.

2. Evaluation of miRNAs expression in SHRP breast cancers

Thirty-six (n=36) SHRP breast cancers were enrolled to the study [18 ER(+)/PgR(–) and 18 ER(–)/PgR(+)]. The cases were matched according to their clinicopathological features (patient age, grade, HER2 status, Ki67 index). Total RNA was isolated from archival formalin-fixed paraffin-embedded (FFPE) tissue blocks with the use of RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Extracted RNA was subjected to miRNA expression profiling with nCounter Human v3 miRNA Expression Assay (NanoString Technologies, Seattle, WA, USA) in Clinical Research Centre in Białystok (dr. Magdalena Niemira and dr. Anna Szałkowska). For each analyzed sample, correction and normalization were performed using nSolver

4.0 software. Moreover, to validate the obtained results, 67 cases of SHRP breast cancers from the TCGA database were analyzed [12]. In the next step, differentially expressed miRNAs between ER(–)/PgR(+) and ER(–)/PgR(+) groups in relation to their HER2 status were identified. miRNET 2.0 database was employed to identify the target genes of selected miRNAs in mammary gland tissue [13].

Moreover, functional annotation analysis (Gene Ontology biological processes, GO BP) using the Functional Annotation Tool by DAVID Bioinformatics Resources 6.81) was performed [14-15].

3. Reevaluation of ER(–)/PgR(+) breast cancer

One hundred thirty-five (n=135) breast cancers primarily designated as ER(–)/PgR(+) were included in the study. FFPE tissue blocks consisting of breast cancer tissue derived from 86 core needle or vacuum-assisted breast biopsies and 76 surgeries. In 27 cases both biopsy and post-surgical material were available. In the next step, the tissues were stained with three anti-ER antibody clones (1D5, Dako; EP1, Dako; SP1, Roche) and one anti-PgR antibody clone (636, Dako) according to the manufacturers' instructions. Subsequently, the evaluation of the obtained stainings was performed. In cases designated as ER(–)/PgR(–) additional staining with the second clone of the anti-PgR antibody was performed (1E2, Roche).

Only cases with ≥1% stained tumor nuclei were regarded as positive for a given receptor according to American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) criteria [16].

4. Statistics

Statistical analyses were performed with the use of Statistica 13 (Tibco, CA, USA), and R statistical environment [17]. Categorical variables were compared by Fisher's exact test or Chi-square test with Yates correction. The normal distribution of the data was assessed using the Shapiro–Wilk test. Continuous variables were analyzed utilizing the Mann–Whitney U test, Kruskal–Wallis test, Student t-test, or two-way ANOVA, when applicable. Correlations between continuous variables were assessed with Spearman or Pearson tests when applicable.

Kaplan–Meier curves were plotted to calculate the survival rates. Hazard ratios (HRs) with confidence intervals (CIs) were estimated using Cox regression analysis.

A *p*-value ≤0.05 was considered significant. In the case of multiple comparisons, Benjamini-Hochberg correction was implemented.

V. DESCRIPTION OF THE PUBLICATIONS INCLUDED IN THE DOCTORAL THESIS

My doctoral thesis consists of four articles (two reviews and two original articles) published in international journals indexed in the Philadelphia List (Master Journal List). The articles focus on SHRP breast cancers – ER(–)/PgR(+) and ER(+)/PgR(–). A special emphasis is put on their pathogenesis, molecular features, and clinicopathological characteristics.

Publication 1.

Kunc M, Biernat W, Senkus-Konefka E.: *Estrogen receptor-negative progesterone receptor-positive breast cancer - "Nobody's land" or just an artifact?* Cancer Treatment Reviews. 2018; 67:78-87.

ER(–)/PgR(+) breast cancers constitute the smallest yet very controversial group of breast malignancies. Due to the very low frequency of these tumors, little is known about their etiopathogenesis, molecular profiles, and clinicopathological features (Figure 1.). In our article, we performed an extensive literature review to summarize the current state of the knowledge in ER(–)/PgR(+) breast cancer research.

The paper starts with a discussion on the leading hypotheses aiming to explain the mechanism of ER(–)/PgR(+) breast cancer development, including pre-analytical, analytical (inadequate fixation, immunohistochemical artifacts), tumor heterogeneity, variable criteria for positivity, the influence of other steroid receptors, growth factors and miRNAs. Molecular features of ER(–)/PgR(+) breast cancer were also discussed.

In the next section, we describe the epidemiology and clinicopathological features of ER(–)/PgR(+) breast cancer. Some data show that ER(–)/PgR(+) breast cancers tend to occur in younger females and are relatively more common in African Americans when compared to ER(+)/PgR(+) breast cancer. Moreover, they frequently demonstrate high-grade histology, high KI67 index, and display basal mRNA profile.

Most studies indicate that patients with ER(–)/PgR(+) phenotype have intermediate overall survival (OS) and disease-free survival (DFS) in between double-positive and double-negative tumors. In general ER(–)/PgR(+), breast cancers show relatively low sensitivity

to endocrine treatment and high sensitivity to chemotherapy. Planning of systemic treatment may be facilitated with the assessment of molecular signatures, e.g. PAM50.

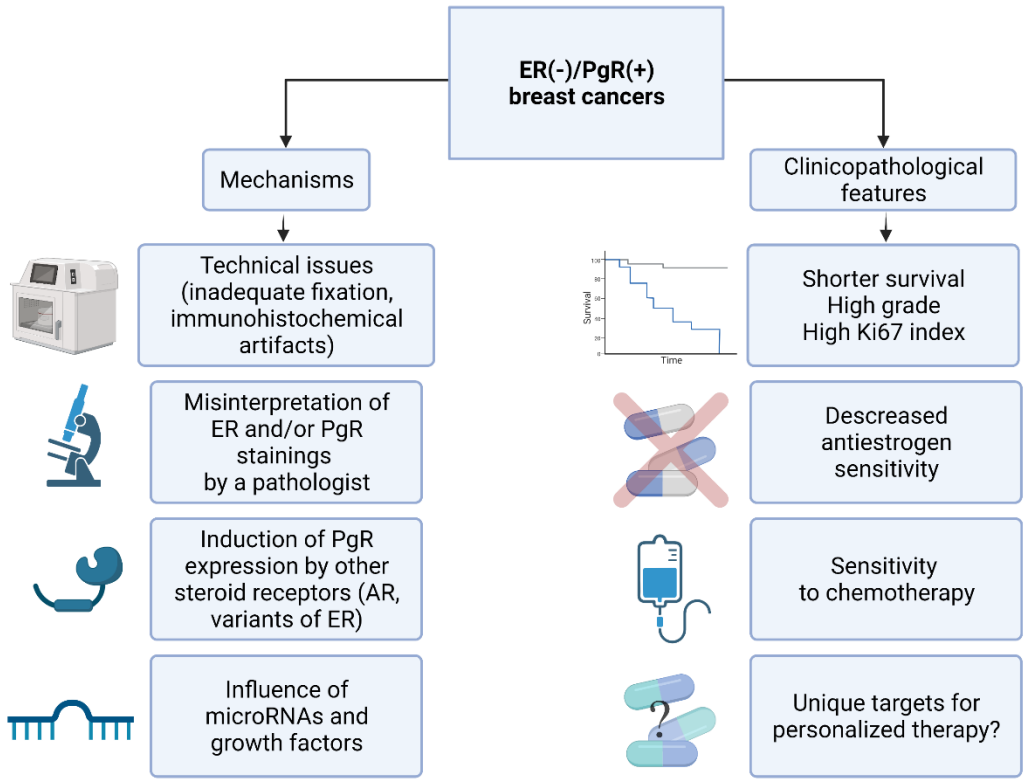


Figure 1. Summary of mechanisms and clinicopathological features of ER(–)/PgR(+) breast cancers.

Publication 2.

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. 2021; 23;13(19):4755.

Clinicopathological characteristics of ER(+)/PgR(–) breast cancers are well established but their pathogenesis and molecular features remain the subject of intensive research. In this paper, we focused on the advances in understanding the mechanisms and biological consequences of PgR loss in ER(+) breast cancer (Figure 2.).

In the first section, we discuss the mechanisms of PgR loss in breast cancer including the loss at the genetic level (e.g. copy number loss, mutations, epigenetic modifications), transcriptional level (e.g. miRNAs), or post-translational modifications

(e.g. phosphorylation, methylation, sumoylation). Lack of PgR expression may be observed in the primary tumor or be a result of clonal selection. A mechanism responsible for PgR loss in ER(+) cancers may be of extreme biological importance. Indeed, some breast cancers with a very high PgR transcriptional activity may not display PgR expression in immunohistochemistry due to the rapid degradation of receptors in proteasomes. This phenomenon is especially common in tumors with growth factors receptors overexpression, e.g. HER2, IGFR, FGFR2. We emphasize that such cases may be paradoxically potentially sensitive to antiprogestins.

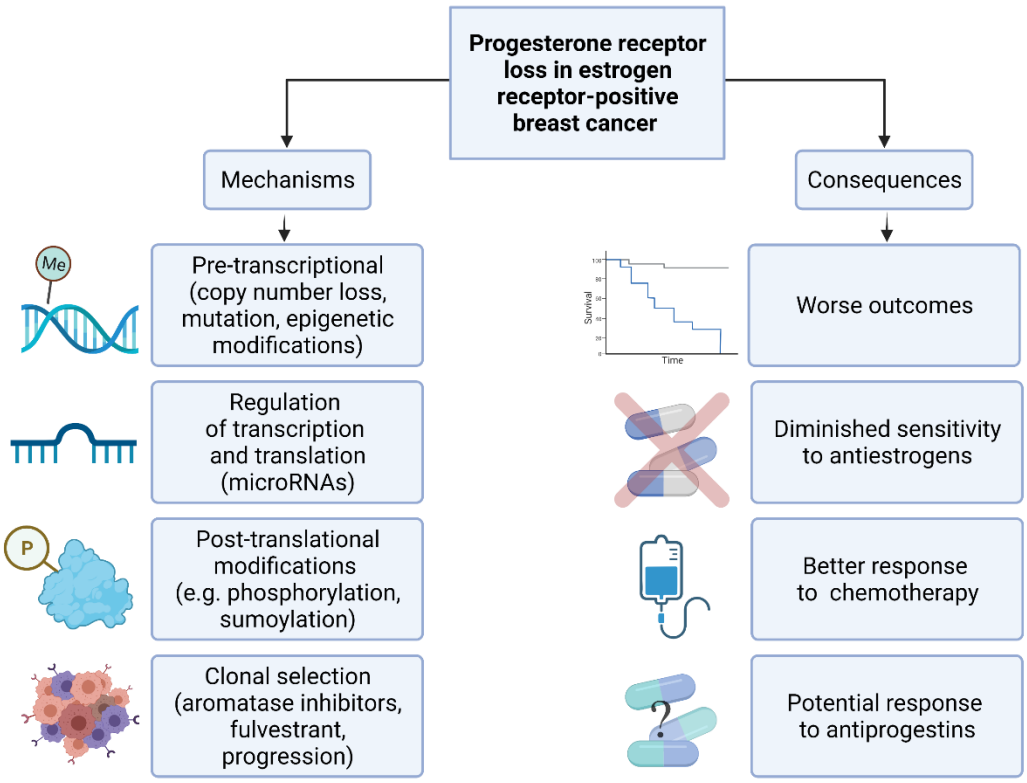


Figure 2. Summary of mechanisms and consequences of PgR loss in ER(+) breast cancers.

In the next part, we focus on PgR loss due to treatment or relapse. Loss of PgR expression in ER(+) tumors may indicate the clonal selection of cells that are resistant to endocrine therapy or chemotherapy and is associated with a worse prognosis.

Moreover, we discuss the genetic landscape and biology of breast cancers with PgR loss. We comprehensively present interactions between ER and PgR in ER(+)/PgR(+) cells and the consequences of PgR loss with a special focus on the potential role of other receptors.

Publication 3.

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. 2020; 20;10(9):617.

miRNAs are short, noncoding oligonucleotides involved in the regulation of transcription and translation. They may play various roles during cancerogenesis and act as tumor suppressors (so-called tsmiRs) or oncogenes (so-called oncomiRs). Some miRNA signatures have prognostic, predictive, and diagnostic values in breast cancer. Our study focused on miRNA profiling of SHRP breast cancer, i.e. ER(+)/PgR(–) and ER(–)/PgR(+). To our knowledge, this is the very first study to investigate this issue.

The study included 18 ER(+)/PgR(–) and 18 ER(–)/PgR(+) breast cancers with available FFPE tissue blocks and known HER2 status. Following quality control of raw data, 4 cases were excluded from analysis, thus the final study group counted 14 ER(+)/PgR(−) and 18 ER(−)/PgR(+) cases. After correction for multiple comparisons, we did not find a miRNA signature differentiating between ER(−)/PgR(+) and ER(+)/PgR(−) breast cancers. However, a trend for differing expression of 8 miRNA was observed. ER(+)/PgR(−) cases tended to express higher levels of miRNAs associated with ER-positivity (miR-30a-5p, miR-29c-3p, miR-141-3p), whereas ER(−)/PgR(+) cancers showed elevated levels of miRNAs characteristic for double- and triple-negative tumors (miR-92a-3p, miR-424-5p). For one of the miRNAs—miR-29c-3p—the association with the ER(+)/PgR(−) phenotype was confirmed in the TCGA cohort ($p = 0.024$; t-test). HER2 status in our cohort was related to significant differences in 33 miRNAs expression levels. The association with HER2 status was confirmed in the TCGA cohort for four miRNAs (miR-1180-3p, miR-223-3p, miR-30d-5p, and miR-195-5p).

Publication 4.

Kunc M, Pęksa R, Cserni G, Iżycka-Świeszewska E, Łacko A, Radecka B, Braun M, Pikiel J, Litwiniuk M, Pogoda K, Szwajkosz A, Biernat W, Senkus E. High expression of progesterone receptor may be an adverse prognostic factor in oestrogen receptor-negative/progesterone receptor-positive breast cancer: results of comprehensive re-evaluation of multi-institutional case series. Pathology. 2022; 21;S0031-3025(21)00542-0; in press

The study aimed to validate diagnoses of ER(–)/PgR(+) breast cancer collected from several Polish and Hungarian centers. We retrospectively enrolled 135 cases. We performed immunohistochemical stainings with three anti-ER antibody clones and two anti-PgR antibody clones. The general agreement in binary classification into negative or positive expression across three investigated clones was substantial (Fleiss' kappa 0.73). Overall, discordant stainings were present in 21 (15.5%) of tumors. The worst concordance was observed between the SP1 and EP1 clones. We also observed discrepancies in PgR staining. Of 42 PgR(–) cases by 636 clones, staining with 1E2 clone demonstrated positive nuclear reaction in 32 (76.2%).

Finally, we confirmed ER(–)/PgR(+) phenotypes in 76 cases (56.3%). Forty-seven (34.8%) cases were rediagnosed as ER(+)/PgR(+), and 12 (8.9%) as ER(–)/PgR(–) (Figure 3.). Confirmed ER(–)/PgR(+) cases were characterized by high-grade histology and very high proliferation index Ki67.

In the group of HER2(–) tumors, cases rediagnosed as ER(+)/PgR(+) with all three anti-ER antibody clones showed the best OS. Confirmed ER(–)/PgR(+) cases, triple-negative, and ER(+) with discordant results of ER stainings across anti-ER antibody clones were characterized by the inferior OS. Subsequently, we investigated if levels of PgR expression influence outcomes in ER(–)/PgR(+)/HER2(–) tumors. Surprisingly, we observed that tumors with PgR expression in >20% of cancer cells were associated with the significantly shorter OS when compared to the group with PgR expression in <20%. The prognostic significance of PgR was retained in multivariate analysis adjusted by stage (HR 5.0, 95% CI 1.3-19.2, p=0.019).

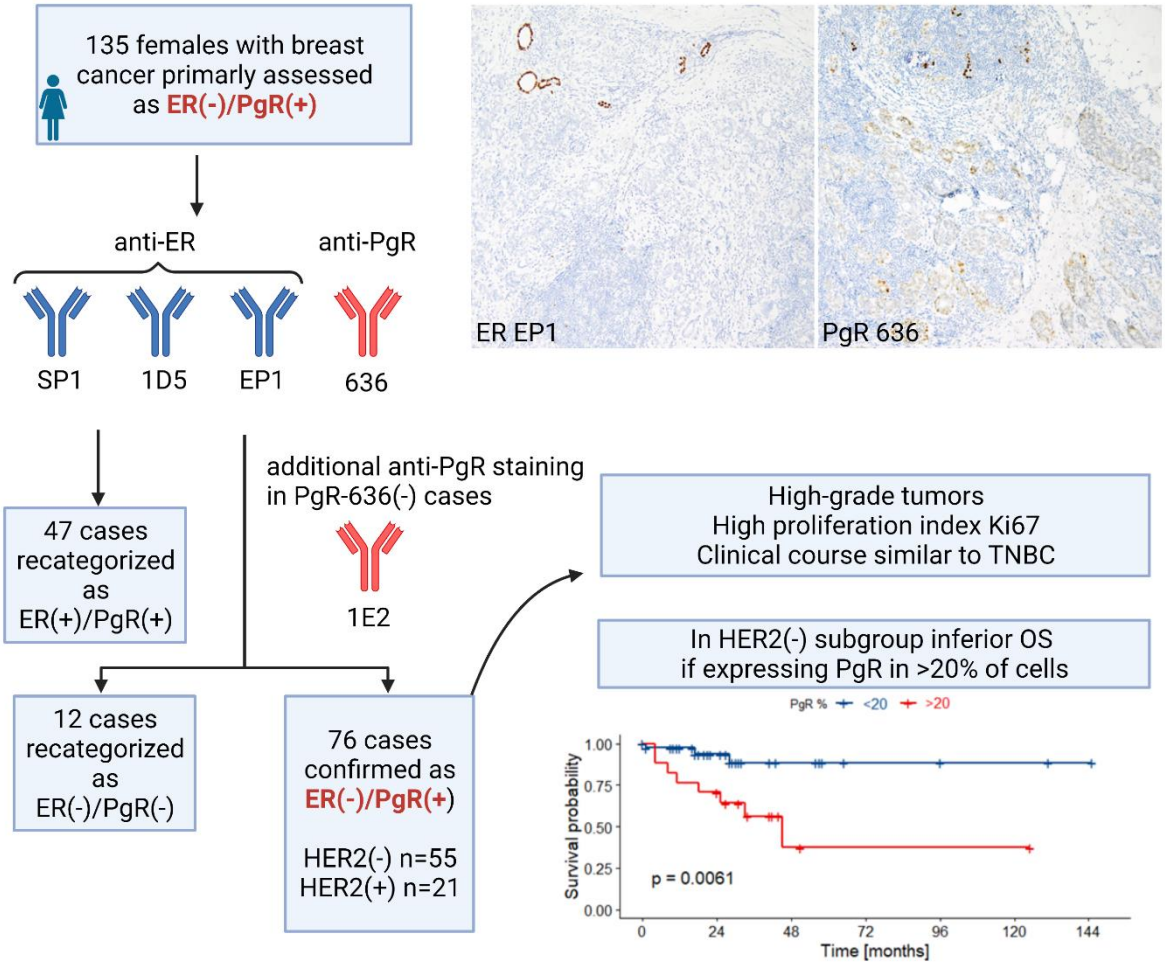


Figure 3. The flow-chart of ER(–)/PgR(+) breast cancers reevaluation. Microscopic pictures display a representative example of confirmed ER(–)/PgR(+) breast cancer with internal positive control.

VI. SUMMARY

The immunohistochemical evaluation of steroid hormones receptors' expression enables the assessment of the prognosis and treatment planning of breast cancer patients. In my dissertation, I summarized the current state of knowledge regarding ER(–)/PgR(+) and ER(+)/PgR(–) breast cancers. In our review articles, we demonstrated that SHRP breast cancers have multiple features distinctive from double-positive and double-negative tumors. Their unique phenotype may make them more resistant to endocrine treatment and more sensitive to chemotherapy.

Moreover, we analyzed miRNA profiles of SHRP breast cancer. We demonstrated that their miRNA signatures are mainly dependent on HER2 status. Nevertheless, we observed the relationships between several miRNAs and ER/PgR status. Our research is the foundation for future translational research on the association between miRNAs and steroid hormones receptors expression in breast cancer.

Finally, we demonstrated that a subset of ER(–)/PgR(+) breast cancers constitute a real and unique group of malignancies characterized by high-grade histology and a high Ki67 index. Moreover, we demonstrated that in the group of ER(–)/PgR(+)/HER2(–) tumors PgR expression in >20% of cancer cells is associated with shorter OS. It undermines the current paradigm according to which higher expression of steroid hormones receptors in breast cancer is generally associated with better outcomes.

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PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ
/
MANUSCRIPTS INCLUDED IN THE DOCTORAL DISSERTATION

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Controversy

Estrogen receptor-negative progesterone receptor-positive breast cancer – “Nobody's land“ or just an artifact?

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ABSTRACT

The estrogen receptor α (ER) and the progesterone receptor (PgR) are one of the most important prognostic and predictive immunohistochemical markers in breast cancer. Breast cancers may express various profiles of hormone receptors: ER(+)/PgR(+), ER(−)/PgR(−), ER(+)/PgR(−) and ER(−)/PgR(+). The existence of the latter profile is a matter of controversy since PgR expressions is induced by ER-dependent pathways in breast cancer cells. One of the most extensively propagated hypotheses trying to explain the origin of ER(−)/PgR(+) breast cancers claims that they are technical artifacts dependent on the immunohistochemical procedure. On the other hand, in recent years there is a growing body of evidence, suggesting that such cancers create a unique group with distinct molecular and clinical features. In the following review, we present background theories on the ER(−)/PgR(+) breast cancer origin and their epidemiological and clinicopathological characteristics, including the predictive and prognostic significance of these rare tumors.

Introduction

The St. Gallen surrogate classification for intrinsic breast cancer subtypes defines four entities: luminal-A-like, luminal-B-like, HER2-positive and basal-like [1]. They are assessed by the immunohistochemical evaluation of estrogen receptor α (ERα – later also referred to as ER), progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2) and Ki-67. Triple-negative breast cancer is closely related to the basal intrinsic phenotype and is characterized by lack of expression of ER, PgR and no overexpression of HER2. Luminal tumors are defined as ER and/or PgR positive, therefore, such tumors may have three distinct profiles: ER(+)/PgR(+), ER(+)/PgR(−), ER(−)/PgR(+). Luminal A-like tumors are characterized by a high expression of ER and/or PgR, whereas luminal B-like cases demonstrate a lower expression of hormone receptors and a higher proliferation rate. Prat et al. suggested the cut-off of > 20% PgR expressing cells best correlates with luminal-A phenotype. Until the 2015 edition, the ER(−)/PgR(+) phenotype had not been included in the surrogate definitions of intrinsic subtypes of breast cancer proposed by the St. Gallen consensus and in the 2017 guidelines the subtype allocation of ER(−)/PgR(+) cancers between luminal A-like and luminal B-like phenotype is still not clearly defined [2]. The use of the reference method, gene-expression profiling (Prediction Analysis of Microarray 50, PAM50) showed that these tumors are mostly basal-like (50–60%) and luminal-A (15–30%), suggesting a significant molecular heterogeneity within the group [3–5]. Extensive research has shown that PgR expression is dependent on ER activity [6]. Therefore, the ER(−)/PgR(+) profile in breast cancer is hard to explain on biological grounds and for this reason some pathologists and oncologists put its existence into question. On the other hand, in recent years there is growing body of evidence that such cancers create a unique group with distinct molecular and clinical features.

In 2004 Olivotto started a debate on the significance of PgR expression evaluation in breast cancer patients. He declared that PgR testing in breast cancer management should be discontinued, due to its negligible role in altering therapeutic decisions [7]. This article has aroused many controversies and initiated worldwide discussion. In response, some authors have raised an important issue: PgR status in ER(−) tumors may provide an important predictive information and PgR positivity may indicate which patients are more likely to respond to adjuvant endocrine treatment [8]. Others pointed out the prognostic value of PgR expression in breast cancer, especially if determined by appropriate immunohistochemical methods [9].

This paper aims to present the possible origin, epidemiology and prognostic/predictive significance of the ER(−)/PgR(+) breast cancer phenotype.

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Table 1
Main studies addressing ER(−)/PgR(+) breast cancers.

Author and year	Population	Method	Total cases	Number of ER(−)/PgR(+) (%)	Cut-off	Mean age	Prognostic significance	Response to endocrine therapy	Clinicopathological features
Keshgegian 1994 [74]	US women	LBA	319	18 (5,6)	–	56	High recurrence rate, CRD 3.5x higher than ER(+)/PgR(+) cases	–	Tumor size, grade, and S-phase fraction higher than in ER(+)/PgR(+) tumors
Bernoux 1998 [46]	Operable primary breast cancer	LBA	3000	62 (2)	> 15 fmol/mg protein	49.4	Trend for worse DFS and the metastasis-free survival than in ER(−)/PgR(+) patients	–	Smaller size and more frequently grade I than ER(−)/PgR(−) tumors
Bardou 2003 [58]	PP and SPORE databases	LBA	14,183	298 (2)	–	–	Probably better prognosis than ER(−)/PgR(−) cancers	Probably worse response to tamoxifen than ER(+)/PgR(+)	–
Olivotto 2004 [7]	Victoria, British Columbia	IHC (ER 6F11, PgR 1A6)	192	0 (0)	–	–	–	–	–
Dowsett 2005 [78]	ATAC Trial	IHC	9336	220 (2.4)	Allred score > 2	–	–	Slight benefit from endocrine therapy	–
Grann 2005 [38]	SEER	Various	155,890	5165 (3.3)	Various	–	Higher CRD than patients in the other groups	–	–
Nadji 2005 [17]	South Florida and Latin America	IHC (ER 1D5, PgR 636)	5993	0 (0)	Any positive nuclear reaction	–	–	–	–
Dowsett 2006 [66]	NATO & CRC study	IHC (ER 1D5, PgR 1A6)	813	26 (3)	H-score: ER > 1, PgR > 19	–	–	Substantial benefit from tamoxifen	–
Kiani 2006 [11]	Pakistanian women (Karachi)	IHC (ER 1D5, PgR IA6)	1625	132 (8.12)	H-score > 74	47.04	–	–	More poorly differentiated and larger tumors than in ER(+) PgR(+)
Viale 2007 [42]	BIG 1–98	LBA IHC	6291	8 (0.1)	LBA: > 10 fmol/mg IHC: > 1%	–	–	–	–
Rakha 2007 [30]	Nottingham Tenovus Primary Breast Carcinoma Series	IHC	1944	60 (3.4)	> 1%	48 (median)		Similar response to endocrine therapy as ER(+)/PgR(−)	Larger tumor size, higher expression of p53, P-cadherin, basal CKs and HER-2 compared to ER(+)/PgR(+)
Dunnwald 2007 [52]	SEER	Various	155,175	4896 (3)	Various	–	Increased risk of mortality compared to ER(+)/PgR(+) patients (particularly high if tumor size > 5 cm or high grade)	–	–
Bird 2008 [45]	Kenyan women (Kijabe)	IHC	129	12 (10%)	> 1%	–	–	–	–
Yu 2008 [51]	Chinese women (Shanghai)	IHC	1863	205 (11%)	> 10%	50	Independent prognostic factor for DFS and OS only for axillary node (+)	Less benefits from adjuvant tamoxifen than ER(+)/PgR(+).	No significant differences in stage, pathologic pattern and HER-2 status when compared to ER(+)/PgR(+)
De Maeyer 2008 [18]	Belgian women, primary operable breast cancers	IHC (ER SP1, PgR SP2)	2013	0 (0)	Any nuclear staining	–	–	–	–
Rhodes 2009 [50]	UK women	IHC	4053	131 (3.2)	Various	–	–	–	–
Stuart-Harris 2009 [59]	Metastatic breast cancers from trials: ANA, AR/BC2, AR/BC3	LBA IHC	979	40 (4.1)	Various	–	Median OS worse than in ER(+)/PgR(+), but similar to ER(+)/PgR(−)	No differences in benefit from an AI or a non AI treatment	–
Liu 2010 [56]	Invasive early breast cancer (British Columbia)	IHC	4046	166 (4.1)	> 1%	–	5 year BCSS higher in ER(−)/PgR(+) than in double negative (non-significant at 10 years)	–	–
EBCTCG 2011 [67]	EBCTCG meta-analysis of 20 trials of 5 years of adjuvant tamoxifen	LBA	21,457	1236 (5.8)	> 10 fmol/mg	–	–	No benefit from TAM in ER poor breast cancer, irrespective of PgR	–
Yi 2011 [75]	US women	IHC	3726	93 (2.5)	> 10%	–	5 years DSS worse than all other subgroups	–	–

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Table 1 (*continued*)

Author and year	Population	Method	Total cases	Number of ER(−)/PgR(+) (%)	Cut-off	Mean age	Prognostic significance	Response to endocrine therapy	Clinicopathological features
Cserni 2011 [40]	Hungarian women	IHC	6587	1* (~0)	> 1%	–	–	–	–
Maleki 2012 [41]	Iranian women	IHC	2432	0* (0)	Any nuclear staining	–	–	–	–
Yang 2012 [60]	Taiwanese women	IHC: ER SP1, PgR SP2	1260	128 (10)	> 10%	53.19	Better OS than double negative patients, but worse than all other subgroups	TAM increased OS and DFS in the lower grade group	–
Ng 2012 [48]	Malaysian women	IHC: ER 1D5, PgR 636)	2629	122 (4.6)	–	48	–	–	More likely to be associated with grade 3 tumors compared to ER(+)/PgR(+) tumors
Park 2013 [54]	The Severance Hospital breast cancer registry	IHC	1180	63 (5.3)	–	45.4	Stage II and III disease: significantly decreased OS, compared to double positive cases	–	Higher proportion of grade 3 tumors and higher prevalence of Ki-67 > 14% than ER(+)/PgR(+) or ER(+)/PR(−) tumors.
	The Korean Breast Cancer Society registration program	IHC	9916	936 (9.4)	–	46.6			
Hefti 2013 [36]	GEM dataset	Gene microarrays	1752	36 (2)			PgR mRNA expression not associated with prognosis in ER-breast cancer	–	–
	NHS dataset	IHC (ER 1D5, PgR 636)	2011	71 (4)			–	–	–
Itoh 2014 [3]	A biomarker discovery study at MD Anderson Cancer Center in Houston	IHC (ER 6F11, PgR 1A6)	501	20** (4)	> 1%	–	The RFS equivalent to ER(+) and better than triple-negative	Low predicted hormone sensitivity by the SET gene signature, but relatively high predicted chemotherapy sensitivity by the DLDA30 predictor	
Ng 2014 [63]	Malaysian women	IHC: ER 1D5, PgR 636)	1992	101 (5.1%)	> 10%	48.5		Identical survival advantage with adjuvant TAM as in ER(+)/PgR(+)	More likely to be grade 3 tumors, compared to ER(+)/PgR(+);more likely to be associated with invasive lobular histology than ER(−)/PgR(−)
Chan 2015 [47]	Spanish women	IHC (Dako ER/PgR pharmDx Kit)	815	56 (6.8)	> 10%	50 (median)	TTR intermediate between ER(+) and ER(−)/PgR(−) tumors (significantly worse than ER + tumors). OS similar to ER + tumors and better than that of ER(−)/PgR(−) tumors.	–	Associated with higher grade and with HER2 overexpression
Fan 2015 [53]	Chinese women	IHC (ER SP1, PgR 1E2)	3966	273 (6.9 – HER2(−) cases)	> 1%	–	No differences in RFS and OS compared to triple negative	Better RFS and OS on adjuvant endocrine treatment	Lower tumor grade and smaller tumor size than triple negative
Yu 2015 [5]	SEER, FDUSCC, publicly available cases	Various	71,590	648 (0.9)	Various	–	Survival outcomes midway between ER(+)/PgR(+) and ER(−)/PgR(−), but the survival curve was more similar to that of the ER(−)/PgR(−) cases.	Lower endocrine therapy sensitivity score of basal-like ER(−)/PgR(+,) compared with luminal-like ER(−)/PgR(+)	Larger tumor size, higher positive node rate, and higher grade compared with ER(+)/PgR(+)
Bae 2015 [62]	Korean women	IHC	6980	90 (1.3)	Allred score > 2	–	Increased risk of recurrence and death compared with ER(+)/PgR(+) tumor in HER2(−) patients. In HER2(+) patients the DFS and OS of not significantly different from ER(−)/PgR(−) tumors		Higher grade and expression of Ki-67, p53, EGFR and HER2 than ER(+)/PgR(+) tumors, similar HER2 overexpression to ER(−)/PgR(−) tumors
Shen 2015 [44]	USA women	IHC: ER 6F11, SP1; PgR 1A6, 1E2	5374	121 (2.3)	> 1%	52.5	Shorter survival than ER(+)/PgR(+)	Shorter DFS than ER(+)/PgR(+), but similar to ER(+)/PgR(−) cases	Higher grade, compared to ER(+)/PgR(−) and ER(+)/PgR(+)
		IHC	13,689	646 (4.1)	> 1%	–			–

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Table 1 (*continued*)

Author and year	Population	Method	Total cases	Number of ER (−)/PgR(+) (%)	Cut-off	Mean age	Prognostic significance	Response to endocrine therapy	Clinicopathological features
Schroth 2016 [4]	Onkologischer Schwerpunkt (OSP) Stuttgart						Improved 10-year survival for stage I cancers compared to the ER(+)/PgR(+)	Shorter survival in patients treated with endocrine agents compared to their non-treated counterparts	
Liu 2016 [76]	Invasive lobular breast cancer (NCDB database)	–	43,230	585 (1.4)	–	–	Relatively constant hazard ratios for OS in patients below the age of 70; in contrast outcomes of ER(+)/PgR(+) cases were the best in patients diagnosed at age 40–59	–	–
Ahmed 2017 [77]	Singapore women	IHC: ER SP1; PgR SP2	8315	92 (1.1)	> 1%	32	A trend for an early recurrence and poorer overall survival as compared with the patients with ER(+)/PgR(+) tumors and similar to ER(−)/PgR(−) tumors	–	More poorly differentiated and larger tumors than double-positive cases

Abbreviations: AI – aromatase inhibitors, BCSS – breast cancer specific survival, CK – cytokeratin, CRD – cancer-related death, DFS – disease-free survival, DSS – disease specific survival, EBCTCG – Early Breast Cancer Trialists Group, ER – estrogen receptor, FDUSCC – Fudan University Shanghai Cancer Center, IHC – immunohistochemistry, LBA – ligand binding assay, mRNA – messenger RNA, NDCB – National Cancer Database, OS – overall survival, PgR – progesterone receptor, RFS – relapse-free survival, SEER – Surveillance, Epidemiology, and End Results Program, TAM – tamoxifen, TTR – time to recurrence, UK – United Kingdom, US – United States.

* Significant reduction in frequency of ER(−)/PgR(+) cases after central reevaluation of locally classified cases.

** 5 cases reclassified after genetic analysis with use of the Affymetrix U133A gene chip.

Materials and methods

A MEDLINE search was performed to identify all original and review articles addressing ER(−)/PgR(+) breast cancer. The following keywords were included: breast cancer, estrogen receptor, progesterone receptor. The analysis was restricted to the time period between 1990 and 2017. Bibliographies, references and related articles were also reviewed. Relevant papers were selected by one author (MK) with the agreement of the expert panel of coauthors. The main findings reported by these studies are shown in Table 1.

Background theories on ER-negative PgR-positive breast cancer existence

As PgR expression is ER dependent, PgR positivity may indicate active ER signaling in breast cancer and, thus, be a marker of potential sensitivity to endocrine treatment. The historical technique for assessing the ER in breast cancer, the ligand-binding assay, relied on determining the formation of ligand-receptor complexes. Therefore, early hypotheses stated that ER(−)/PgR(+) breast cancers might have resulted from high levels of endogenous or exogenous estrogens and subsequent ER saturation or competition with exogenous ligands in the binding assay [10,11]. Higher levels of circulating estrogens in younger patients could potentially explain the younger age of patients diagnosed with ER(−)/PgR(+) cancer as highlighted by several studies. Some other possibilities included mutated ER, which had lost its ability to bind ligands, but remained capable of activating pathways inducing PgR expression. Nevertheless, nowadays immunohistochemistry, which assesses the presence of ER protein irrespective of its ligand-binding status has replaced the binding assay in hormone receptor determination in breast cancer, and it still has not eliminated the ER(−)/PgR(+) subgroup. On the other hand, one of the most extensively propagated hypotheses trying to explain the origin of ER(−)/PgR(+) breast cancers claims that they are technical artifacts dependent on the immunohistochemical procedure. In his article from 2008 Allred blew the whistle on severe improprieties in immunohistochemical evaluation of ER in Canada [12]. Alarmingly, nearly 40% of the 2000 primarily ER(−) samples from Newfoundland and Labrador turned out to be ER(+) after being reevaluated in Ontario. There are several potential pitfalls regarding immunohistochemical evaluation of hormone receptors (Table 2).

Fixation issues seem to be of fundamental importance. Both prolonged and shortened formalin fixation may potentially decrease the hormone receptor immunoreactivity. However, in the case of prolonged fixation this effect appears after a very long period of time, and the results immunohistochemical stains of specimen fixed for as much as 96-h (e.g., because of weekend or holiday break in laboratory work) are still reliable [13]. In contrast, nowadays due to intensive schedules and demands from clinicians and patients, fixation times are frequently inadequate. Goldstein et al. demonstrated that in samples fixed for less than 6 h, the ER Q-score was significantly decreased [14]. Delayed formalin fixation, another common problem related to logistics of sample handling, had a similar effect on both ER and PgR immunoreactivity, but its level varied with the antibody clones used [15]. As a result of these problems, especially weakly ER(+) but strongly PgR(+) cases may easily be misdiagnosed as ER(−)/PgR(+) cases if improperly fixed.

Of note, in a recent study comparing three commercial ER assays available for autostainer vendors: Dako, Leica, and Ventana, poor reproducibility in ER(−)/PgR(+) phenotype was observed [16]. ER(−)/PgR(+) breast cancers constituted 1.2% of cases when evaluated by Leica system, and only 0.5% while utilizing the Ventana and Dako systems. More intriguingly, not a single ER(−)/PgR(+) case was concordant across all three assays. Importantly the authors used various antibodies (1D5 mixed with ER-2-123 in Dako, 6F11 in Leica, SP1 in Ventana) recognizing different ER regions. Utilizing 1D5 antibody is associated with an “all-or-none” phenomenon: the samples are either

diffusely positive or completely negative. 1D5 is capable of binding to both intact ER, as well as ligand independent but still functional ER splicing variants [17]. Interestingly, Nadji et al. reported that assessment of ER in 5993 breast cancer cases using only 1D5 showed no ER (−)/PgR(+) samples [17]. De Maeyer emphasized that application of low sensitivity IHC techniques may lead to erroneous results in particular in certain groups of patients: younger patients, those with high-grade tumors, and/or HER2(+) tumors [18].

Inter- and intraobserver variability often interferes with the pathological diagnostic process and the assessment of hormone receptors in breast cancer is not an exception. Interobserver concordance for hormone receptor scoring varies slightly amongst studies, but is commonly reported as high [16,19,20]. Nevertheless, Reisenbichler et al. has noted that the discordance rate in reporting hormone receptor scoring in cases with low expression of ER is non-negligible (up to 5%) [19]. Hence, this variability may influence ER(−)/PgR(+) frequency amongst medical centers. To minimalize the interobserver variability, all pathologists should follow the widely accepted American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) criteria for IHC assessment of hormonal receptors, which designates the stain "positive" when at least 1% of tumor cell nuclei are immunoreactive [21].

ER(−)/PgR(+) breast cancer tissue, especially when assessed by biopsy or in tissue microarray, may reflect the heterogeneity of neoplastic cells with only some populations expressing this unique phenotype within a tumor, that also contains double-negative or double-positive cells. This hypothesis has never been verified, but Torhorst et al. observed intratumoral heterogeneity of ER expression in 8.8% and of PgR in 28.9% of breast cancer samples evaluated using tissue microarrays [22].

Besides the hypotheses considering ER(−)/PgR(+) tumors artifacts, biological explanations of "true" ER(−)/PgR(+) breast cancer origin are possible. As mentioned before, PgR expression is dependent on ERα activity, but may also occur independently. In pregnant mice the concentration of mammary ERα is low but levels of ERβ and PgR are high, suggesting that, under certain conditions, PgR is up-regulated by ERβ [23]. This is further supported by studies conducted on the alpha ERKO mouse, which lacks ERα expression and still maintains synthesis of PgR [24]. Nevertheless, in human breast cancer cells ERβ represses activation of the *PGR* gene [25], thus, further research is needed to fully explain the role of ERβ in ER(−)/PgR(+) tumors. Moreover, even sex hormone receptor splice variants may influence tumor biology in different ways. For example, the levels of ERβΔ5 show an inverse correlation with PgR expression, and therefore may be potentially involved in the pathogenesis of ER(−)/PgR(+)-breast cancer [26]. Several splice variants of hormone receptors were reported in ER(−)/PgR(+) breast cancers, including exon 5 lacking variant of ER, which is ligand-independent and difficult to detect using IHC [27].

Table 2
Possible immunohistochemistry-related explanations of ER(−)/PgR(+) phenotype origin. For details see text.

Phase	Problem
Tissue preparation	Delayed or inadequate fixation of tissue – ER may be more labile than PgR, thus delayed fixation in formalin may result in rapid degradation of ER with relatively spared PgR (less than 8 h of fixation in 10% formalin resulting in ER being washed out during the dehydration period) Antigen-retrieval procedures that inadequately re-expose proteins masked during fixation
Staining	Use of antibodies which recognize different epitopes Lack of negative and/or positive control Intratumoral heterogeneity of ER and PgR expression
Evaluation of staining	Too high definitions of positivity (> 10% rather than > 1%) Intra- or interobserver variability

Another receptor which may be of importance in ER(−)/PgR(+) breast cancer is the androgen receptor (AR). Patients with AR(+) tumors have better outcomes and response to adjuvant endocrine therapy [28]. Higa et al. hypothesized that some tumors expressed AR and only minute amounts of ER [described in pathology reports as ER(−)/AR(+)] [25]. The androgen binding to these receptors may potentially trigger not only proliferation, but also transcription of *PGR* in ER(−)/PgR(+) breast cancers. In this scenario AR antagonists (enzalutamide and bicalutamide) could potentially be useful in the treatment of patients expressing the ER(−)/PgR(+) phenotype [29]. Surprisingly, in one study, AR was not expressed more often in ER(−)/PgR(+) tumors than in ER(+)/PgR(−) cases [30].

Furthermore, we cannot exclude the existence of ER-independent pathways that activate PgR expression in breast cancer. For example IGF-I and EGF have been shown to up-regulate PgR expression. Borras et al., confirmed this phenomenon by developing the ER(−)/PgR(+) Evsa-T mammary cell line, obtained after long term incubation of a double-negative cell line in appropriate growth factors, which resulted in the selection of this unique clone [10].

Another postulated explanation relates to a missense ER mutation, Tyr537Asn, isolated from a cell line obtained from metastatic breast cancer, which was ER(−) by ligand-binding assay. The affected region encodes ligand-binding domain, but Tyr537Asn is capable of activating the estrogen-dependent pathway even in the absence of a ligand [31]. Thus, some "technically" ER(−) cancers (i.e. tumors with mutations influencing antibody- or ligand-binding sites) could maintain potent hormone-independent ER activity and still induce PgR expression.

Another molecule potentially involved in the pathomechanism of ER(−)/PgR(+) tumors is the high mobility group A1 (HMGA1) – a chromatin-associated protein involved in many intracellular processes related to cancer progression. In breast cancer, HMGA1 overexpression correlates positively with PgR and HER2 expression, whereas negatively with ER levels [32]. Interestingly, HMGA1 regulates microRNA (miRNA) expression in various tumors and we hypothesize that miRNA plays a crucial role in the pathogenesis of single hormone receptor positive breast cancer. MiRNAs are short fragments of ribonucleic acid consisting of 19–22 nucleotides, which negatively regulate gene expression through the promotion of mRNA degradation or by direct inhibition of translation. In breast cancer some miRNAs are up-regulated, while others are down-regulated. Changes in miRNA expression are involved in initiation of carcinogenesis, tumor progression, metastasis formation and drugs resistance [33]. The signatures of 4 miRNAs, (miR-520g, miR-377, miR-527-518a and miR-520f-520c) predict PgR expression, whereas miR-342, miR-299, miR-217, miR-190, miR-135b and miR-218 are associated with ER expression [34]. Certain miRNAs: miR-221/222, miR-206, miR-18a, and miR-22 are able to interfere with ER expression or regulate ER-dependent pathways, possibly participating in the formation ER(−)/PgR(+) tumors [35]. Detection of miRNA profiles associated with ER(−)/PgR(+) breast cancer could give an insight into the biology of these tumors and help in the precise identification of the true sex hormone receptor phenotype.

Molecular characteristics of ER(−)/PgR(+) breast carcinomas have been poorly defined so far. Nevertheless, there are a few studies addressing this issue. Hefti and colleagues analysed 20 breast cancer datasets with integrated gene expression microarray and clinicopathological data [36]. According to their findings, up to 97% of originally (information from the medical record) immunohistochemically determined ER(−)/PgR(+) cases were reclassified as ER(+) or PgR(−) after genetic analysis. The authors claimed that this subtype is non-reproducible and probably is not a distinct biological entity. However, a few cases held this profile even after molecular analysis. On the other hand, two more recent studies have obtained the opposite results. Hormone receptor status in a series of stage I-III breast cancers was assessed by both IHC and the Affymetrix U133A gene chip [3]. Amongst 20 immunohistochemically ER(−)/PgR(+) cancers only 5 (25%) were reclassified as ER(+) due to the gene

expression profile. Interestingly, all remaining ER(−)/PgR(+) cases were of non-luminal subtypes. Similar results were obtained by Yu et al.: 5 of 17 (29%) cases turned out to have high expression of *ESR1* (gene encoding for ER*α* alpha) mRNA, whereas the remaining 12 cases were characterized by low *ESR1* and variable *PGR* mRNA [5].

The existence of the discussed phenotype is strongly supported by the discovery of 59 genes uniquely expressed by ER(−)/PgR(+) breast cancers [4]. Genes significantly enriched in ER(−)/PgR(+) breast cancers include those related to the estrogen signaling pathway, branched-chain aminoacids degradation, fatty acid degradation, prolactin signaling, thyroid hormone synthesis and dopaminergic synapse. Interestingly, *ESR1* and *GATA3*, crucial elements of estrogen signaling were up-regulated in ER(−)/PgR(+) cancers when compared to double negative tumors and down-regulated relative to double positive ones. This expression pattern clearly shows intermediate position of ER(−)/PgR(+) breast cancer, such a phenomenon may reflect residual ER expression which could not be identified by relatively low sensitivity tools like IHC. A similar phenomenon may exist in the subgroup of triple-negative breast cancers termed the Luminal/Androgen Receptor subtype, demonstrating activation of ER, AR, prolactin, and ErbB4 signaling, but ER(−) in IHC [37].

Epidemiological and clinicopathological characteristic

The relative frequency of ER(−)/PgR(+) varies amongst studies from 0% to approximately 10%, but these differences are at least partially dependent on various cut-offs for ER and PgR positivity (> 1% vs > 10%). Amongst women registered in the SEER database between 1990 and 2000, the proportion of this phenotype dropped from 4.5% to 1.7% (Fig. 1) [38], possibly as a result of the application of more reliable immunohistochemical techniques. In one study all cases diagnosed as ER(−)/PgR(+) by IHC method of the 90s converted to ER(+) using current IHC method [39]. Three groups reported a significant reduction in the frequency of ER(−)/PgR(+) tumors after a central reevaluation of locally classified cases [40–42]. In Hungarian and Iranian studies, only in one of the 182 reviewed cases and in none of the 43 tumors respectively, the original phenotype remained unchanged [38,39]. It may reflect the mentioned false-negative results due to immunohistochemical staining or high interobserver variability between local pathologists and experts from tertiary care centers. Nevertheless, other authors have reported much higher prevalence of ER(−)/PgR(+) cases. Even when using optimal fixation methods and any level of staining as a cut-off value for positive results, ~5% of all cases were still designated as ER(−)/PgR(+) [43].

The frequency of this phenotype is possibly modified by racial and geographic factors, as the Caucasian to African American ratio of

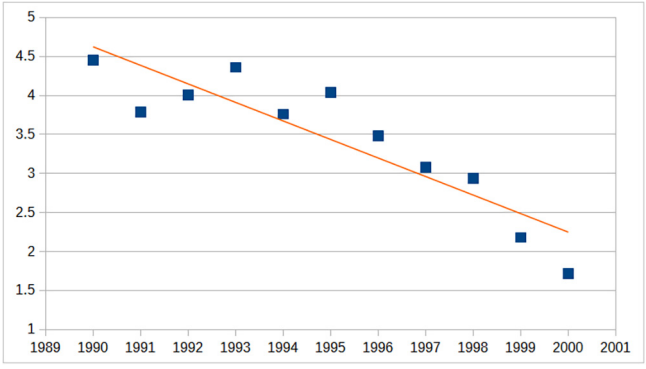

Fig. 1. The percentage (blue squares) and linear trend line (orange) of frequency of ER(−)/PgR(+) in the SEER database between 1990 and 2000. The data is taken from Grann et al. [36]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

patients with ER(−)/PgR(+) breast cancer was significantly lower than in ER(+) tumors [44]. In Kenya the hormone receptor status distribution is skewed toward ER(−) cases [including 10% which are PgR(+)]; this phenotype is generally more common in younger patients [45]. The majority of studies indicates that age at diagnosis in the ER(−)/PgR(+) group tends to be lower, compared to women with other cancer phenotypes [11,44,46–51]. Age was also an important modifier of clinical outcomes, with older patients (especially > 65 years of age) with single-receptor positive breast cancer having significantly higher hazard ratios for breast cancer mortality [52]. The differences in the number of ER(−)/PgR(+) cases amongst certain age groups indicate that this hormonal status defines a distinct clinicopathological entity – especially as Rhodes et al. restricted their study group to cases assessed by laboratories with optimal performance in a national quality-assurance program [50].

ER(−)/PgR(+) cancers tended to be more often poorly differentiated and larger than double-positive cases, but opposite associations were observed when matched with double-negative tumors [47,53]. Most of the discussed tumors were of the invasive non-special type (“ductal”) histology, but they manifested invasive lobular histology more frequently than ER(−)/PgR(−) cases [48]. The separation into intrinsic subtypes by PAM50 test revealed that the vast majority of cases were of the basal subtype (53–65%), followed by the luminal A subtype (15–27%) [3–5]. It indicates that majority of ER(−)/PgR(+) breast cancers share molecular features with triple negative cases, which are most frequently of the basal subtype.

In the majority of studies no significant differences in the frequency of regional lymph node involvement were found when compared to other breast cancer subtypes [11,47,54]. The pattern of distant metastases was similar to triple-negative cases: visceral and lung metastases were more common, whereas skeletal involvement was less likely [53].

The data regarding the expression of other immunohistochemical biomarkers is sparse when ER(−)/PgR(+) breast cancer is concerned. When compared to ER(+) cases they tended to be associated with markers of poorer prognosis: p53 expression, basal cytokeratin expression, reduced expression of E-cadherin and negative expression of androgen receptor [30]. Proliferation marker Ki-67 showed higher immunohistochemical scores, as well as higher *MKI67* mRNA expression in ER(−)/PgR(+) tumors compared to double-positive cases [3,54]. HER2 amplification was more likely to occur in single-receptor positive tumors, than in double positive cases, hence partially explaining the worse prognosis of ER(−)/PgR(+) cancers. On the other hand, no difference was identified when compared with the double negative phenotype [47,49].

Predictive and prognostic significance of ER-negative PgR-positive phenotype

The discussion about the role of PgR expression in predicting response to adjuvant therapy in breast cancer is ongoing. In this paragraph we rather focus on the prognostic and predictive value of joint ER and PgR status, specifically ER(−)/PgR(+) phenotype.

Prognostic significance

Studies conducted in the 1990s demonstrated poorer prognoses in ER(−)/PgR(+) cancer than in double-positive tumors. Keshgegian and Cnaan reported 3.5 times higher cancer-related death rates in the former group [55]. Another study compared ER(−)/PgR(+) with a double-negative cohort, showing a trend toward a better prognosis in the former, but without a statistical significance at 10 year follow-up [46]. These findings were confirmed in a more recent study [56]. On the other hand, Serbian authors observed significantly shorter disease-free survival of patients with ER(−)/PgR(+) carcinomas compared to the other steroid hormone receptor phenotypes [57]. Analysis of two big databases: PP and SPORE, published in 2003, generated inconclusive results regarding ER(−)/PgR(+) cancer, because of a small number of events in this group [58]. Nevertheless, some trends could be

observed in patients who received no systemic therapy. The lowest risk for recurrence and death was noted in patients with ER(−)/PgR(+) tumors. Unfortunately, the opposite trend was observed in patients with adjuvant treatment. This phenomenon may at least partially be explained by a selection bias related to omitting adjuvant therapies only in the lowest risk group. An investigation of 205,736 patients from the Surveillance, Epidemiology, and End Results (SEER) Program registry diagnosed between 1990 and 2000 showed no differences between stage I patients with double-positive and ER(−)/PgR(+) tumors in terms of all-cause mortality, but the latter had worse disease specific mortality [38]. Surprisingly, a recent German study of > 15,000 breast cancer patients showed that ER(−)/PgR(+) breast cancer patients had the longest median OS, especially in stage I. Rakha et al. found that patients diagnosed with both subtypes of single receptor-positive breast cancer had comparable OS, intermediate between double-negative and double-positive tumors, but in terms of DFS ER(−)/PgR(+) patients resembled those with ER(−)/PgR(−) cancer [30]. Similar conclusions regarding OS of both single hormone receptor-positive phenotypes were drawn from an analysis of three randomized phase III trials of aromatase inhibitors [59] and recently from four large datasets [5]. In turn, similar DFS and OS were observed in ER(−)/PgR(+) and double receptor-negative Taiwanese breast cancer patients [60].

Liu et al. observed relatively constant hazard ratios for OS in single hormone receptor positive cases in patients below the age of 70; in contrast outcomes of ER(+)/PgR(+) cases were the best in patients diagnosed at age 40–59 [56]. On the other hand, other authors demonstrated that age of patients may divide ER(−)/PgR(+) patients into two prognostic subgroups: young women (40–55 years old) had much worse survival, whereas the > 55 years population had only a slightly poorer outcome than the double-positive patients [51]. However, among ER(−)/PgR(+) cancers only the lymph node status was an independent prognostic factor for DFS/OS. It is further supported by findings from a study limited to ER(+) and/or PgR(+), and HER2(+) cancer, which highlighted that amongst node-negative breast cancer patients the outcomes were similar irrespective of hormone receptor status [54]. On the contrary, lymph node-positive patients with single hormone receptor positive tumors had worse prognosis than double receptor-positive women.

Parise and Caggiano have subdivided ER(−)/PgR(+) breast cancer cases into HER2(+) and HER2(−) and demonstrated that both groups had survival similar to other subtypes of ER(−) breast cancer [61]. In another study, in the presence of HER2 overexpression, the ER and PgR expression pattern was not associated with differences in survival, but in HER2(−) cancer single hormone receptor cases had significantly poorer outcomes than double positive ones; moreover they tended to manifest with high Ki-67 level and high expression of EGFR and p53 [62]. Furthermore, patients diagnosed with ER(−)/PgR(+) tumors had the most dismal prognosis amongst all the subtypes irrespective of Ki-67 levels, which suggests that this phenotype exemplifies a distinct biological entity. Somewhat perplexing conclusions were drawn from the Malaysian study, in which no differences in survival between ER(−)/PgR(+) and double hormone receptor positive breast cancers were found [63]. Interestingly, ER(+)/PgR(−) cancers in this study had poorer survival than ER(−)/PgR(+) cancers. An analogous trend was noted by Shen et al. but only in the subset of patients receiving endocrine therapy, which may correspond to PgR being a surrogate marker of the activity of estrogen dependent pathways even without detectable ER expression [44]. One study aimed to assess the influence of the semiquantitative level of PgR positivity on prognosis of ER(−)/PgR(+)/HER2(−) breast cancer and it was correlated with neither OS nor DFS [53]. On the contrary, Park et al. found that a higher PgR expression level corresponded with a better outcome [64].

The timing of initial recurrence showed characteristic patterns: ER(−)/PgR(+) and double-negative tumors had higher rates of relapse early in the follow-up, whereas ER(+) cases had a rather low annual risk of recurrence, which remained relatively constant for many years [47].

To summarize, it is difficult to draw definitive conclusions regarding the prognostic significance of the ER(−)/PgR(+) phenotype. It may stem from the small number of cases or differences in diagnostic techniques resulting in comparisons of unequal subgroups, but can also reflect geographical differences in the biology of this breast cancer phenotype. Nevertheless, the recent 8th edition of the AJCC Cancer Staging Manual, has incorporated the biomarkers into staging, producing 120 categories of patients with distinctive prognosis [65]. In several scenarios PgR positivity in ER(−) cancers down-stages the disease, emphasizing better outcomes in some groups of ER(−)/PgR(+) cancers when compared to double-negative ones with similar TNM.

Predictive significance

Predicting response to adjuvant endocrine treatment is essential for the proper management of breast cancer patients. Since epidemiological data suggest that patients with ER(−)/PgR(+) cancers tend to be premenopausal, it is especially important to determine tamoxifen sensitivity in these patients [51].

Early reports have suggested that locally advanced and metastatic ER(−)/PgR(+) breast cancers have a poorer response to endocrine therapy than double-positive cases, but in a more recent series of metastatic breast cancer no significant differences in the clinical benefits were found between these groups [59].

In the analysis of two databases Bardou and colleagues claimed that ER(−)/PgR(+) breast cancer is probably less sensitive to adjuvant endocrine therapy, but definitive conclusions were difficult to draw because of the small number of events [58]. Nevertheless, in some other studies positive PgR status in the ER(−) group was associated with a slight benefit from tamoxifen treatment [42,56,66]. Fan et al. clearly demonstrated that in ER(−)/PgR(+)/HER2(−) group, patients treated with adjuvant endocrine therapy had significantly longer DFS and OS than those without endocrine therapy [53]. However, Early Breast Cancer Trialists' Collaborative Group performed a meta-analysis of 20 trials in early breast cancer of about 5 years of tamoxifen versus no adjuvant tamoxifen. Perplexingly they reported that in ER poor breast cancer PgR evaluation had not identified any subgroup with significant benefit [67].

In a Chinese cohort, ER(−)/PgR(+) breast cancer patients derived less advantage from adjuvant endocrine therapy than double-positive cases; the opposite was observed for adjuvant chemotherapy [51]. The same study indicated that a CMF (cyclophosphamide, methotrexate and 5-fluoruorouracil) regimen in ER(−)/PgR(+) breast cancer is more effective than CA(E)F (cyclophosphamide, doxorubicin/epirubicin and 5-fluoruorouracil). Tumor grade may be an important factor stratifying the ER(−)/PgR(+) cancers into low-grade tamoxifen-responsive and high-grade tamoxifen-unresponsive groups [60]. In turn, some authors observed identical survival benefits from adjuvant tamoxifen in PgR(+) tumors irrespective of ER status, whereas ER(+)/PgR(−) patients benefited less [27,48]. The results of the study by Schroth and colleagues may be somewhat confusing [4]. They observed worse outcomes of ER(−)/PgR(+) patients with endocrine treatment compared to those without endocrine therapy. However, in multivariate analysis this phenomenon was restricted to stage I patients receiving tamoxifen, irrespective of tumor grade and other factors.

The general trends described above have been supported in molecular studies: 90% of ER(−)/PgR(+) tumors are characterized by low predicted endocrine sensitivity by the sensitivity to endocrine therapy (SET) gene signature, but relatively high predicted chemotherapy sensitivity by the diagonal linear discriminant analysis 30 (DLDA30) predictor [3]. These findings suggest that in the ER(−)/PgR(+) subset of patients rational treatment would be a combination of endocrine therapy and chemotherapy. Actually, in common clinical practice up to 80% of such patients receive both chemotherapy and endocrine therapy [62]. Another molecular study revealed that patients with PAM50 identified luminal ER(−)/PgR(+) tumors benefited from endocrine

therapy, whereas basal cancers did not [5]. It was further supported by a higher ER group score (which reflects hormonal sensitivity) in the luminal cases.

Several hypotheses aim to explain tamoxifen effectiveness in ER(−)/PgR(+) tumors. First of all it may be due to false-negative results of ER staining. If it is not the case, tamoxifen may exert some other biological ER-independent effects, e.g., inhibition of calmodulin-mediated process, protein kinase C activity, reducing IGF-1 levels, increasing intratumoral TGF*α*, AMPK activation and metabolic reprogramming [68]. Some studies indicate that the ERβ isoform, which is not routinely assessed in breast cancer, may be responsible for the response observed in some triple negative and ER(−)/PgR(+) cases [69]. However, this does not explain why PgR(+) patients benefit more compared to triple negative ones. Possibly, signaling pathways downstream of PgR may interact with and improve ER-independent tamoxifen activity. The responsiveness to tamoxifen of ER(+)/PgR(+) breast cancer may be dependent on PgR isoforms ratio. In this group, high PgR-A to PgR-B ratio was found to be a predictor of poor benefit from endocrine therapy [70]. The value of PgR-A:PgR-B ratio evaluation in ER(−)/PgR(+) cancer is yet to be established.

Recently, Campbell et al. have proposed the combined endocrine receptor (CER), which is calculated basing on both ER and PgR Allred scores, and classifies tumors into three groups: CER negative, impaired and high [71]. The authors suggest that reclassification of a portion of ER(−)/PgR(+) tumors as CER impaired would lead to more patients with single hormone receptor-positive disease to be considered suitable for endocrine treatment. This approach can be easily implemented, but needs verification in larger groups of patients before being put into clinical practice.

Conclusions and future direction

ER(−)/PgR(+) breast cancers have a distinct clinical course, response to treatment, and molecular features when compared to other breast cancer types, however some of them are actually technical artifacts or consequences of too high definitions of positivity. According to the current guidelines published by the ASCO/CAP, every case of ER(−)/PgR(+) requires repeated testing with a separate sample [72]. Moreover, we recommend that any such case should be validated by an experienced pathologist, preferentially from a tertiary care center.

The biology of ER(−)/PgR(+) breast cancers is probably influenced by sex hormone receptor variants which are not routinely evaluated in clinical practice (e.g. splice variants of ERα, ER*β* and AR). Presumably, some miRNAs interfere with transcription and translation of hormone receptor coding genes, thus participating in the pathogenesis of single hormone receptor-positive breast cancers. Hopefully, new therapeutic strategies will emerge, including effective modulation of PgR, AR, and splicing variants of ER, or silencing of miRNA. Thus, further studies concerning the molecular pathogenesis and biology of ER(−)/PgR(+) breast cancer are strongly recommended.

Since these cancers respond to both chemotherapy and endocrine therapy, both should be considered in their treatment schedules. Currently, the 2017 St Gallen consensus and the 2017 National Comprehensive Cancer Network guidelines consider the ER(−)/PgR(+) phenotype equivalent to other hormone receptor-positive tumors [2,73]. Concluding from the above mentioned studies, such approach may be beneficial for some women, although a significant portion of patients may not respond to endocrine treatment. Thus, it may be worthwhile to consider prediction of benefit from endocrine therapy and chemotherapy in ER(−)/PgR(+) cases using gene signatures, especially in “high-risk” patients (intermediate/high tumor burden, high Ki-67, nodal involvement). Since gene signatures are relatively expensive and not generally accessible, expression of surrogate IHC markers (TFF1/CK5/EGFR) proposed by Yu et al. in ER(−)/PgR(+) breast cancer and high tumor grade may potentially designate hormone unresponsive ER(−)/PgR(+) patients [5]. Finally, the institutions which are not routinely performing PgR evaluation should consider this at least in ER(−) tumors to identify patients who can possibly benefit from anti-estrogen treatment.

Conflict of interest

The authors declared that there is no conflict of interest.

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Review

Lost but Not Least—Novel Insights into Progesterone Receptor Loss in Estrogen Receptor-Positive Breast Cancer

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Simple Summary: Most breast cancers co-express estrogen receptor α (ERα) and progesterone receptor (PgR). These cancers are sensitive to endocrine therapy and, in general, have superior outcomes. However, a subset of tumors expresses ERα but loses expression of PgR in various mechanisms. The processes driving the loss of PgR may cause resistance to hormonal treatment and a more aggressive clinical course. The current review summarizes current knowledge on the biology of ERα-positive PgR(−)negative breast cancer and discusses the associations between molecular mechanisms and clinical characteristics.

Abstract: Estrogen receptor α (ERα) and progesterone receptor (PgR) are crucial prognostic and predictive biomarkers that are usually co-expressed in breast cancer (BC). However, 12–24% of BCs present ERα(+)/PgR(−) phenotype at immunohistochemical evaluation. In fact, BC may either show primary PgR(−) status (in chemonaïve tumor sample), lose PgR expression during neoadjuvant treatment, or acquire PgR(−) phenotype in local relapse or metastasis. The loss of PgR expression in ERα(+) breast cancer may signify resistance to endocrine therapy and poorer outcomes. On the other hand, ERα(+)/PgR(−) BCs may have a better response to neoadjuvant chemotherapy than double-positive tumors. Loss of PgR expression may be a result of pre-transcriptional alterations (copy number loss, mutation, epigenetic modifications), decreased transcription of the *PGR* gene (e.g., by microRNAs), and post-translational modifications (e.g., phosphorylation, sumoylation). Various processes involved in the down-regulation of PgR have distinct consequences on the biology of cancer cells. Occasionally, negative PgR status detected by immunohistochemical analysis is paradoxically associated with enhanced transcriptional activity of PgR that might be inhibited by antiprogestin treatment. Identification of the mechanism of PgR loss in each patient seems challenging, yet it may provide important information on the biology of the tumor and predict its responsiveness to the therapy.

Keywords: estrogen receptor; progesterone receptor; breast cancer; treatment; microRNA

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1. Introduction

Estrogen receptor α (ERα) and progesterone receptor (PgR) are crucial prognostic and predictive biomarkers in breast cancer (BC). Expression of steroid hormone receptors (HRs) in cancer cells justifies the introduction of endocrine therapies (ET), e.g., selective estrogen receptor modulators (SERMs), aromatase inhibitors (AIs), or selective estrogen receptor degraders (SERDs) [1]. These therapies primarily target ER, but BCs co-expressing PgR tend to show an even better response to hormonal treatment. Since the progesterone receptor gene (*PGR*) is dependent on ERα, the negative PgR status may indicate altered ERα signaling and impaired response to ET [2]. In the last two decades, the prognostic and

predictive value of PgR expression has been widely disputed, with some authors postulating even to abandon PgR evaluation [3,4]. However, expression of *PGR* is included in both the 21-gene recurrence score (Oncotype DX, Genomic Health Inc., Redwood City, CA, USA) and the 50-gene signature classifying BC into the molecular intrinsic subtypes (PAM-50) [5]. Additionally, multiple studies confirmed the usefulness of joint immunohistochemical (IHC) evaluation of ERα, PgR, human epidermal growth factor receptor 2 (HER2), and Ki67, which enables subclassification of BC into surrogate intrinsic phenotypes, with the cut-off value discriminating between luminal A-like and luminal B-like tumors proposed at 20% of cells positive for PgR expression [6]. Nevertheless, according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines, in routine assessment BC is considered PgR(−) if <1% or 0% of tumor cell nuclei are immunoreactive [7].

Single hormone receptor-positive breast cancers have two distinct categories. First, ERα(−)/PgR(+) BC is extraordinarily rare and is molecularly, morphologically, and clinically similar to triple-negative breast cancer [8,9]. Another type, ERα(+)/PgR(−), is relatively more common, constituting approximately 12–24% of all BC cases [10,11]. The prognostic and predictive value of this phenotype has been thoroughly analyzed and several reviews and meta-analyses have been recently published [10,12]. In general, ERα(+)/PgR(−) BCs are more often aggressive, high-grade tumors, with high proliferation index, high glucose metabolism, and outcomes inferior to double-positive tumors [13,14]. Nonetheless, patients with single hormone receptor-positive BC still benefit from hormonal therapy, and recent findings emphasize the importance of ET implementation in this group of patients [15].

ERα(+)/PgR(−) tumors develop more commonly in patients older than 55 years than the double-positive cases [10]. Lower estrogen levels in elderly females may contribute to lower expression of ERα-dependent proteins, e.g., PgR [16]. Moreover, the phase of the menstrual cycle at which the tumor is excised can influence the PgR status: carcinomas removed in the luteal phase more often display PgR(−) phenotype, compared to the follicular phase [17]. Other risk factors for ERα(+)/PgR(−) BC development include hormone replacement therapy (combination of estrogen and synthetic progestin), alcohol consumption, and some antidepressants [18–20].

PgR expression provides independent prognostic information and increases the prognostic accuracy of ER assessment in primary BC [21]. One study reported that the presence of PgR(+) proliferating (Ki67-expressing) cells but not PgR(+) non-proliferating cells is associated with better disease-free survival [22].

However, no effect of PgR expression on the benefit from tamoxifen use was demonstrated in the meta-analysis of 20 trials involving more than 21 thousand early BC patients [23]. In metastatic ER(+) disease, PgR expression is associated with an increased probability of response to tamoxifen, longer time to treatment failure, and longer overall survival [24]. No difference was seen, however, in the magnitude of benefits from the addition of cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitor to ET for advanced BC treatment [25].

On the other hand, PgR-negativity in ERα(+) BC is associated with higher rates of pathological complete response to neoadjuvant chemotherapy (NAC) when compared to double-positive BC [26–28]. Thus, PgR status may be of great importance in predicting response to NAC in ERα(+) patients.

Moreover, PgR is a predictive factor (as depletion of PgR correlates with poor response to megestrol acetate in advanced BC) and a potential target for personalized therapy in BC, either with the use of antiprogestins or, surprisingly, progestogens [29].

While the epidemiology and clinical behavior of this type of single hormone receptor-positive BC is well described, the underlying biology of these tumors remains obscure. In 2005 a comprehensive description of the biology of PgR loss in ERα(+) BC was published by Cui et al. [30]. The current paper aims to provide an update on this subject, focusing on the studies published in the last 15 years. A special emphasis is put on the novel

mechanisms of PgR loss, genetic landscape and biology of ERα(+)/PgR(−) tumors, and the role of microRNA (miRNA) in the down-regulation of PgR.

2. Mechanisms of PgR Negativity

BC may either show primary PgR negative phenotype (i.e., negative PgR expression in tumor sample assessed before systemic therapy), lose PgR expression during neoadjuvant treatment (assessed in the postsurgical specimen), or acquire PgR negative phenotype in local relapse or metastasis.

2.1. Loss of PgR at the Genetic Level

Among the HER2(−) group of tumors, the ERα(+)/PgR(−) cases show significantly lower *PGR* mRNA expression when compared to ER(+)/PgR(+) cancers, suggesting that in most cases the loss of PgR occurs before or during transcription [31]. At the genetic level, PgR loss might be explained by a copy number loss of the *PGR* gene, which was reported to occur in 27–52% of cases of BC [31]. Importantly, exogenous expression of PgR in breast cancer cells ensued growth inhibition in an MCF-7 cell line with a heterozygous loss of the *PGR* gene [32].

On the other hand, *PGR* mutations are exceedingly rare, since in the analysis of 959 ER(+)/PgR(−) cases all the tumors were classified as *PGR*-wild-type [33]. In another large dataset, only 9 missense mutations in the *PGR* gene were identified (estimated frequency 0.36%) [34]. A recent study on *PGR* variants in metastatic ER(+) BC demonstrated that 3 out of 4 samples of functionally deleterious Y890C variant were PgR(−) by IHC, so this specific variant may contribute to PgR loss by clonal selection [35].

2.2. The Interplay between Growth Factors and PgR Expression

The role of growth factors and growth factors receptors in the pathogenesis of ERα(+)/PgR(−) tumors has been postulated for many years [30]. Insulin-like growth factor (IGF), epidermal growth factor (EGF), and heregulin activate signaling pathways down-regulating PgR expression [30]. Accordingly, ERα(+)/PgR(−) BCs demonstrate an increased frequency of epidermal growth factor receptor (EGFR) and HER2 overexpression [30]. In normal circumstances, ERα mainly exerts genomic effects but in the case of enhanced growth factor stimulation, membrane-initiated steroid signaling (MISS) starts to predominate [26]. This transition ensues PgR down-regulation by its phosphorylation via extracellular signal-regulated protein kinase (ERK1/2), phosphatidylinositol 3-kinase (PI3K), Akt, and mammalian target of rapamycin complex 1 (mTORC1) (Figure 1). Importantly, SERMs can stimulate MISS, which partially explains the greater benefits of ERα(+)/PgR(−) patients from AIs treatment compared to tamoxifen [30,36].

Additional proofs of the role of growth factors in the development of ERα(+)/PgR(−) BC come from a neu-related lipocalin-transforming growth factor α (NRL-TGFα) transgenic mouse model [37]. During tumorigenesis, ERα expression was noted in all types of precursor lesions and persisted in cancer, whereas PgR expression was lost very early. In bi-transgenic mice overexpressing prolactin (PRL) and TGFα (NRL-PRL/TGFα), these hormones cooperatively enhance Akt activity, resulting in decreased PgR and increased ERα expression [38]. Despite enhanced ERα expression, the developed tumors were insensitive to estrogens, again supporting the hypothesis on diminished hormone responsiveness in ERα(+)/PgR(−) BC. Thus, targeting growth factors pathways may increase sensitivity to ET in single hormone receptor-positive BC.

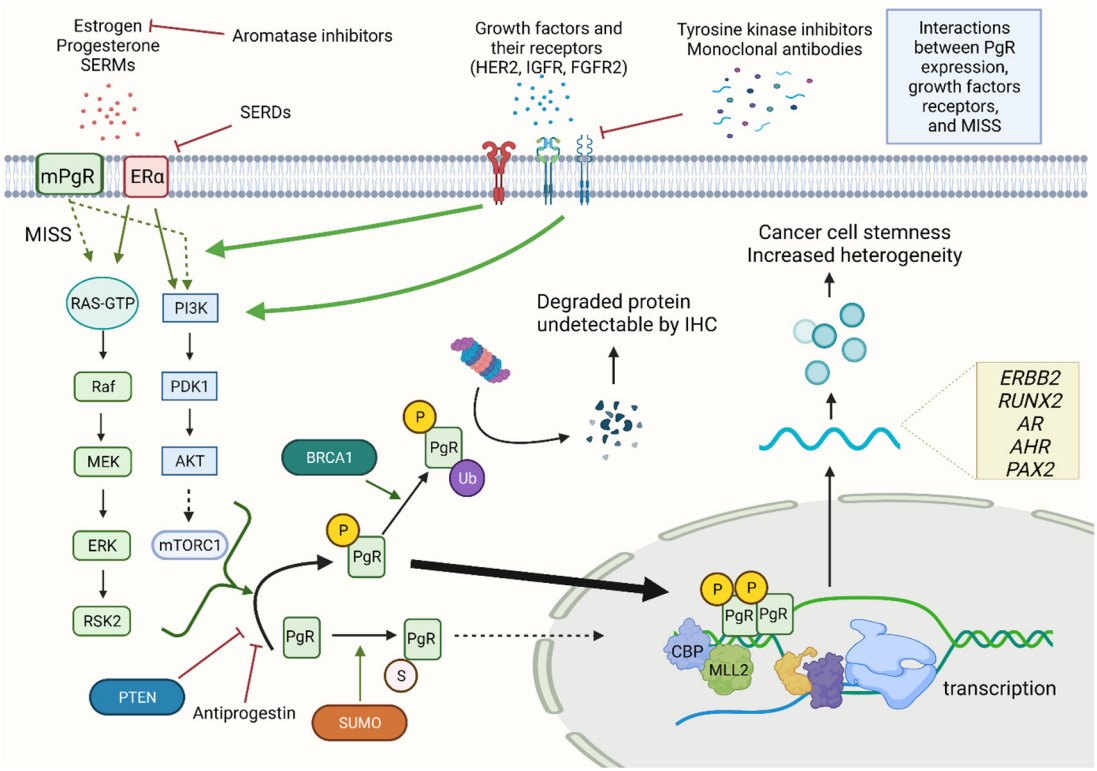

Figure 1. Interactions between PgR, growth factor-dependent signaling and MISS Green arrows demonstrate stimulatory effects, red T-shaped lines depict inhibition. Overactive growth factors receptors stimulate MISS and directly activate various signaling pathways leading to activation of multiple kinases, i.e., ERK, AKT, RSK2, mTORC1, which phosphorylate PgR at Ser294. Phosphorylated PgR is undersumoylated, undergoes rapid ubiquitination and degradation in proteasomes reflected by PgR(−) status in immunohistochemistry. Phosphorylated PgR is also transcriptionally overactive, recruits CBP and MLL2, and enhances transcription of genes involved in cancer progression. Abbreviations: *AHR*—aryl hydrocarbon receptor; AKT—protein kinase B; *AR*—androgen receptor; BRCA1—Breast cancer type 1 susceptibility protein; CBP—CREB-binding protein; ERα—estrogen receptor α; *ERBB2*—Erb-B2 Receptor Tyrosine Kinase 2; ERK—extracellular-regulated kinase; FGFR2—fibroblast growth factor receptor 2; HER2—human epidermal growth factor receptor 2; IGFR—insulin-like growth factor receptor; IHC—immunohistochemistry; MEK—mitogen-activated protein kinase; MISS—membrane-initiated steroid signaling; MLL2—mixed linage leukemia gene 2; mTORC1—mammalian target of rapamycin complex 1; P—phosphate residues; (m)PgR—(membranous) progesterone receptor; *PAX2*—paired box 2; Raf—rapidly accelerated fibrosarcoma; PDK1—3-phosphoinositide-dependent protein kinase-1; PI3K—phosphoinositide 3-kinase; PTEN—phosphatase and tensin homolog; RAS—rat sarcoma virus; RSK2—ribosomal S6 kinase 2; *RUNX2*—RUNX Family Transcription Factor 2; SERDs—selective estrogen receptor degraders; SERMs—selective estrogen receptor modulators; Ub—ubiquitin. Created with BioRender.com—accessed date 22 September 2021.

2.3. Molecular Mechanisms Underlying False-Negative PgR Staining in IHC

Progesterone receptor undergoes multiple post-translational modifications, including phosphorylation, acetylation, sumoylation, methylation, and ubiquitination [39]. Even in the absence of ligands, PgR is constitutively phosphorylated at some sites, and exposure to progestogen results in a net increase in the phosphorylation [40]. The result of this modification depends on a specific phosphorylation site that modulates PgR stability, nuclear transport, DNA binding, and transcriptional activity. Hormone binding results in poly-ubiquitination of PgR leading to ligand-induced PgR down-regulation—this process is paradoxically the hallmark of cells actively expressing PgR-dependent genes [40]. In human BC cells, ERK1/2 activation triggers PgR-B phosphorylation at Ser294, which, thereby, inhibits PgR sumoylation at Lys388. Undersumoylated PgR(−)B is derepressed and transcriptionally overactive, thus highly sensitive to low progestin concentration [41]

(Figure 1). However, Ser294 phosphorylation targets the receptors for rapid proteasomal degradation [42]. Moreover, PgR Ser294 and Ser400 phosphorylation reduce PgR nuclear export, probably enhancing the genomic action of progesterone [43], and phosphorylation-induced PgR desumoylation enhances the transcription of proliferative genes via recruitment of a CREB-binding protein (CBP) and mixed linage leukemia gene 2 (MLL2) [44]. Thus, in the final effect, PgR might express enhanced transcriptional activity but, simultaneously, undergo instant degradation and be undetectable by IHC [42]. An animal study by Zhang et al. demonstrated that the loss of tumor suppressor, Tat-Interacting Protein (Tip30), accelerates cancerogenesis in the MMTV-Neu mouse model of BC, and leads to the development of exclusively ER(+)/PgR(−) tumors [45]. Loss of Tip30 results in impaired degradation of EGFR and enhanced Akt signaling, which correlated with both increased expression and phosphorylation of ERα and loss of PgR in IHC staining [45]. In in vitro culture, the PgR protein was detectable following proteasome inhibition, and the progesterone antagonist RU486 suppressed the growth of Neu+/Tip30−/− tumors [45].

Finally, various clones of anti-ER and anti-PgR antibodies may show discordant results, and multiple additional pre-analytic or analytic factors influence the final quantification of steroid hormones expression. Failure to detect PgR expression by IHC occurs in various laboratories with a frequency of 5 to 15% of cases [46]. While PgR-negativity assessed by IHC may be a technical issue, the other possibility is that alternative splicing of PgR produces cancer-specific variants of PgR that are undetectable with N-terminally targeting antibodies. These truncated variants are generated by the deletion of some of the eight exons of *PGR* or by the preservation of introns and are capable of binding to progesterone, interacting with co-factors, and binding to DNA, thus they may remain functional [47]. Nevertheless, the clinical significance of alternative splicing of PgR needs to be elucidated. Identification of patients with false-negative PgR status may help to identify patients who are more likely to benefit from ET.

2.4. Influence of Tumor Suppressors Loss on PgR Expression

The phosphatase and tensin homolog (PTEN) is a tumor suppressor frequently lost in BC [48]. The role of PTEN is to dephosphorylate phosphatidylinositol 3,4,5-triphosphate (PIP3), thus the loss of PTEN correlates with higher levels of PIP3, which, in turn, activates the Akt signaling pathway [48]. Loss of heterozygosity at the *PTEN* locus coexisting with HER2 overexpression results in substantial Akt activation, leading to loss of PgR [49] (Figure 1). Additionally, PTEN-knockout mice (K8PTEN-KO) demonstrate increased proliferation of mammary epithelial cells mainly restricted to the preferential expansion of PgR(−) cells [50].

In contrast to PTEN, the association between Breast cancer type 1 susceptibility protein (BRCA1) and PgR expression is ambiguous. On the one hand, BRCA1 was reported to stimulate the ubiquitination of PgR protein by E2 enzyme UbcH5c and its subsequent degradation [51]. On the other hand, Sanford et al. found no difference in the proportion of low-positive (<10% positive cells) and negative PgR staining between patients with and without deleterious germline *BRCA1* mutations [52].

2.5. Epigenetic Mechanisms of PgR Suppression

DNA methylation is the most important epigenetic mechanism orchestrating transcription. The first report on the inverse association between *PGR* promoter methylation and PgR expression in BC was published in 1996 and since then this observation has been confirmed by several studies [53]. Recent data demonstrate that IHC PgR(−) tumors show higher *PGR* methylation [54–57]. Nonetheless, in PgR(−) breast tumors, *PGR* methylation is usually either low or absent, so hypermethylation of *PGR* promoter is unlikely the major mechanism of PgR silencing, albeit some data are contradictory [56–58]. Interestingly, one study reported a higher incidence of DNA methylation in *PGR* promoter in HER2-amplified/overexpressing cases, pointing to the role of methylation in the pathogenesis of ER(+)/PgR(−)/HER2(+) breast tumors [59].

Several studies point to an association between *PGR* methylation and patients' outcome, e.g., tamoxifen resistance [57,60]. Additionally, long-term tamoxifen treatment leads to epigenetic silencing of ER-responsive genes, including *PGR* [61]. Owing to a high prevalence of ER(+)PgR(−) phenotype among breast tumors recurring after tamoxifen treatment, *PGR* methylation status was proposed as a predictive marker for tamoxifen insensitivity [61]. Consequently, loss of PgR was also demonstrated in BC cell lines with decreased tamoxifen sensitivity following long-term treatment [62]. Moreover, in MCF-7 BC cell line signaling from membrane-associated ER contributes to epigenetic modulation of *PGR* gene via the action of histone methyltransferase enhancer of Zeste homolog 2-EZH2 [63].

Numerous groups have reported on the restoration of *PGR* gene expression in PgR(−) cell lines following treatment with agents blocking DNA epigenetic modifications, namely the inhibitors of histone deacetylases and DNA methyltransferases [64,65]. Exposure to epigenetic modulators also resulted in increased *PGR* mRNA expression in the hormone-receptor-positive MCF-7 cell line [64]. In the future, it may be possible to convert PgR(−) BC into PgR(+) with the use of epigenetic modulators in order to enhance its sensitivity to ET [66].

2.6. The Interplay between Isoforms and Splice Variants of Steroid Hormone Receptors and PgR Expression

Whereas most estrogenic actions in BC cells seem to be driven by ligand binding to ERα homodimers, the latter may also form heterodimers with ERβ1, which can promote transcription of a distinct pool of genes, and to down-regulate several ERα-dependent genes, including *PGR* (Figure 2) [67,68]. The inverse correlation between ERβcx, a splice variant of ERβ, and PgR was noted; interestingly PgR-low BCs expressing ERβcx showed poorer response to tamoxifen [69].

Expression of PgR is also modulated by splice variants of ERα, e.g., ERα36, which positively correlates with PgR expression [70,71]. In vitro study utilizing ERα36 knock-out cell lines demonstrated reduced levels of PgR and its altered phosphorylation at Ser294 and Ser345 [71].

Additionally, there is a dominant-negative splice variant of ERα (ERαΔ7), which is non-functional, but is detected by IHC. This may explain why a subset of ERα(+) tumors shows the molecular characteristics of the basal subtype [72]. Interestingly, the frequency of PgR expression in ERα(+)/ERαΔ7-high basal carcinomas was 29.7% compared to 85.2% for ERα(+)/ERαΔ7-low luminal B carcinomas [73]. Identification of such hormone receptor variants may in the future support treatment decision-making, but current routine procedures have not incorporated their assessment yet.

2.7. MicroRNA (miRNA) Profiles of ERα (+)/PgR(−) Breast Cancers

miRNAs are small non-coding molecules with an average length of 22 nucleotides [74]. They regulate gene expression via the formation of miRNA-induced silencing complex (miRISC), which binds to the 3'UTR of a target gene [75]. Subsequently, translational repression, mRNA destabilization, degradation, and deadenylation occur [75].

The interplay between miRNAs and ERα expression is well described, but still not completely understood. Estrogens bound to ERα regulate miRNA processing and the formation of miRISC interacting with Drosha, DICER, and protein argonaute-2 (AGO2), and in this way influence gene repression by miRNAs [76]. On the contrary, multiple miRNAs modulate the expression and action of ERα via direct interactions with *ESR1* mRNA and alterations of ERα coregulators. Additionally, some oncogenic miRNAs interfere with ERα-dependent signaling pathways, which, in consequence, may result in partial loss of ERα functionality reflected by loss of PgR expression in BC (i.e., acquisition of ER(+)/PgR(−) phenotype).

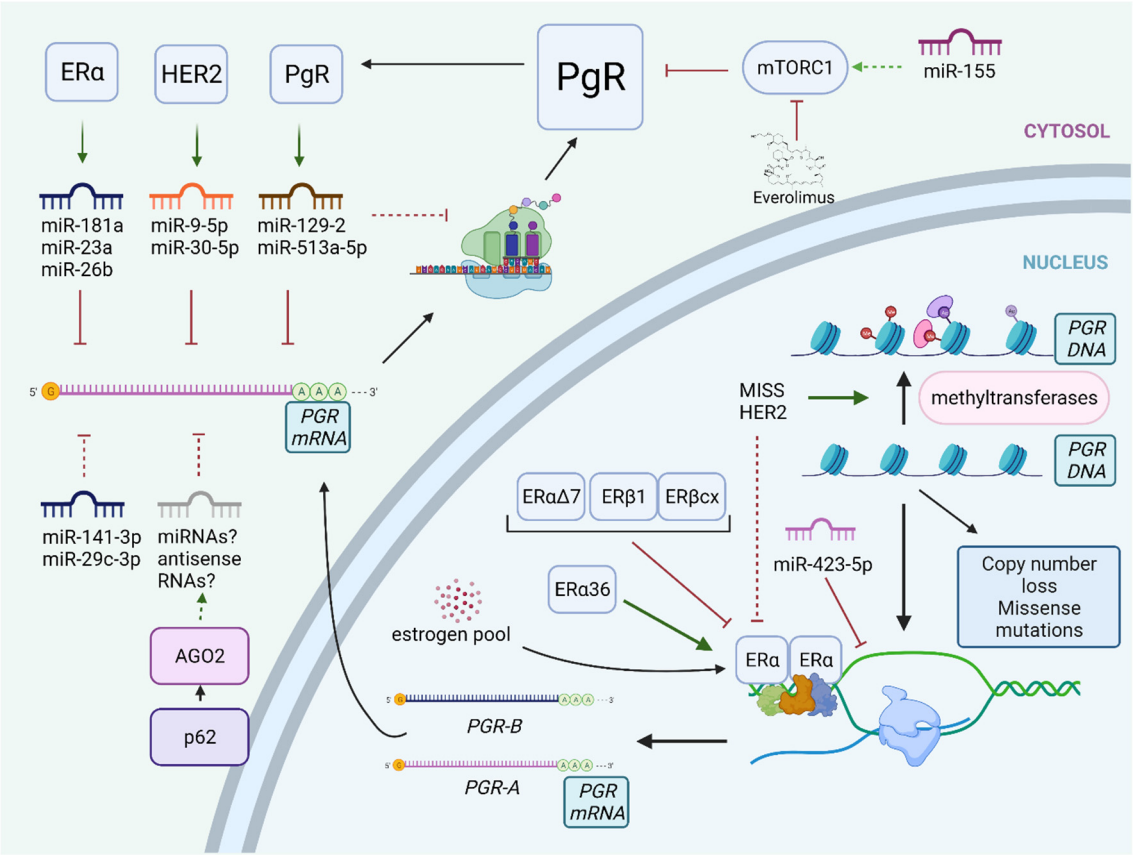

Figure 2. Pre-translational mechanisms of PgR loss and down-regulation. Green arrows indicate stimulatory effects, red T-shaped lines depict inhibitory effects, dotted lines show potential effects. At pre-transcriptional stage, PgR loss is a consequence of methylation of *PGR* promoter, copy number loss (often), or mutations (very rarely). Splice variants of ERα and ERβ may either suppress or activate the transcription of *PGR*. Low levels of estradiol after menopause are frequently insufficient to induce expression of PgR. *PGR* mRNA is a direct target of multiple miRNAs, but some miRNAs may down-regulate PgR indirectly, e.g., via activation of mTORC1. For details, see text. Abbreviations: AGO2—protein argonaute-2; ERα—estrogen receptor α; HER2—human epidermal growth factor receptor 2; miRNAs—microRNAs; MISS—membrane-initiated steroid signaling; mTORC1—mammalian target of rapamycin complex 1; *PGR*—progesterone receptor gene; PgR—progesterone receptor. Created with BioRender.com—accessed date 22 September 2021.

Recent studies have also shed some light on miRNA regulation of PgR expression. Interestingly, the 3′UTR of *PGR* is the longest amongst mRNAs encoding steroid receptors (9434 nucleotides) but surprisingly contains only six conserved miRNA binding sites. It was demonstrated that exogenous miR-423-5p is capable of inhibiting *PGR* gene transcription in vitro [77], miR-126-3p suppresses PgR expression in mouse mammary gland [78], and miR-181a, miR-23a, and miR-26b down-regulate PgR in ERα(+) BC [79,80]. miR-181a and miR-26 are repressed by estrogen and they belong to the feed-forward loop involving ERα. Their down-regulation following estrogenic stimulation leads to *PGR* up-regulation and their up-regulation in ERα(+) tumors may contribute to ERα(+)/PgR(−) BC development [79]. The main interactions between microRNAs and PgR expression are shown in Figure 2.

Estrogen-dependent PgR up-regulation may be abrogated by progestin-controlled miRNAs, most notably miR-129-2 and miR-513a-5p. Progesterone treatment of BC cell lines leads to the up-regulation of miR-129-2, resulting in down-regulation of PgR, and tumors with elevated miR-129-2 have significantly decreased levels of PgR [81]. Similar effects were observed for miR-513a-5p, which represses PgR expression and reduces the amounts of PgR induced by estrogenic stimulation [82]. In vitro studies demonstrate that inhibitors

of miR-129-2 increase expression of PgR providing a potential tool for stabilization of PgR levels in PgR-low/negative patients considered for hormonal therapy [81].

In our recent study, we compared miRNA profiles of two groups of single-steroid-hormone-receptor-positive BC, ER(+)/PgR(−) and ER(−)/PgR(+) [83]. The first group demonstrated elevated levels of miR-30a-5p, miR-29c-3p, miR-141-3p—members of miRNA clusters characterizing ER(+) tumors, and miR-423-5p, whose role in PgR silencing was discussed before [77]. Interestingly, miR-30-5p has previously been shown to suppress PgR expression in BC cell lines [83]. Additionally, the miR-29 family targets and represses transcription of the PgR-regulated gene, *ATP1B1* [82]. Conversely, progestin treatment inhibits the expression of miR-29. miR-141-3p is another miRNA with reciprocal associations with PgR: down-regulation of miR-141-3p increases PgR levels, whereas progestin treatment decreases levels of miR-141-3p [84]. In conclusion, miR-29 and miR-141-3p up-regulation in ER(+)/PgR(−) BC may reflect diminished progestin-dependent signaling in these tumors.

An interesting mechanism of PgR regulation in BC, partially driven by miRNA, involves a model, in which early lesions recapitulate the developmental program of normal mammary gland orchestrated by progesterone signaling via PgR and moderate HER2 expression [85]. This program facilitates the early dissemination of cancer cells, enhancing migration and stemness. Growing lesions gradually increase their tumor cell density and overexpress HER2, which up-regulates the expression of miR-9-5p and miR-30a-5p, leading to the down-regulation of *PGR* in the mouse BC model. This mechanism increases the proliferation of cells contributing to primary tumor growth but impairs its ability to spread. Plausibly, ERα (+)/PgR(−)/HER2(+) BCs show inferior prognosis because they represent an end-point in the pathway beginning with early, occult dissemination initially driven by PgR(+) cells, while clinically overt PgR(−) cancers may comprise only of residual scattered phospho-PgR(+) spots with stem cell potential and an ability to spread [85].

An additional mechanism of PgR regulation by miRNA involves miR-155 and the mTOR pathway. In BC, IGF-mediated mTORC1 activation down-regulates PgR expression [30]. Increased expression of miR-155 in ERα(+) BC cells enhances mTORC1 signaling via inhibition of the mTORC2 signaling component Rictor [86]. TCGA data on BC show that levels of Rictor and PgR positively correlate with each other, whereas Raptor (complexed with mTORC1) shows an inverse correlation with PgR [86]. mTOR inhibitor, everolimus, demonstrated efficacy in combination with ET in advanced BC and is generally believed to reverse endocrine resistance by inhibition of mTORC-1-dependent phosphorylation of ERα, but de-repression of PgR expression may represent another possible mechanism of action [87–89]. Nevertheless, limited data suggest that PgR status is not a predictive factor in advanced/metastatic BC treated with everolimus [90].

Curiously, a group of small duplex RNAs, antigene RNAs (agRNAs) are also able to regulate gene expression by targeting gene promoters (noncoding transcripts). Several studies demonstrated that PgR expression is regulated by synthetic agRNAs mediated by argonaute (AGO) proteins, but it was unknown if similar effects may be mediated by endogenous RNAs [91]. A very recent study shows that sequestosome 1 (p62) accumulation in BC cells triggers PgR suppression in an AGO2-mediated mechanism, comprising most likely agRNAs, not miRNAs [92]. On the contrary, in another study, high AGO2 levels were correlated with PgR loss due to altered ERα signaling probably driven by miRNA [93]. If small RNAs can precisely up-regulate expression PgR in BC to increase its sensitivity to ET remains to be elucidated.

3. Loss of PgR during Therapy and in Breast Cancer Relapse

A large meta-analysis of steroid HRs discordance in primary and recurrent BCs estimated the frequency of secondary PgR loss at 46% of patients, being more common in distant metastases than in local relapses [23]. The prognostic significance of this conversion is not well established, however, some studies report on the association between worse outcomes and the negative conversion of steroid HRs [12]. The loss of ERα and/or PgR in relapsing tumors or after primary systemic treatment probably indicates the selection

of HR-negative cells in a heterogeneous pool of tumor cells. Moreover, circulating tumor cells (CTCs) frequently show discordant profiles with primary tumors. PgR(−) CTCs are present in 68–87% of patients with PgR(+) primary tumor, and this pool may be responsible for ERα(+)/PgR(−) metastases [94]. On the other hand, in metastatic BC, the loss of PgR expression on CTCs may occur, even if still present in both primary tumors and metastases [95].

The switch from PgR(+) to PgR(−) after neoadjuvant chemotherapy occurs in 12–15% of cases and is associated with worse clinical outcomes [96,97]. Similarly, neoadjuvant ET with SERMs or AIs may lead to the down-regulation of ERα and PgR, respectively [12]. A letrozole-induced decrease in PgR expression is most likely due to decreased estrogens levels and diminished estrogenic signaling [98,99]. Accordingly, studies on patient-derived xenografts and cell lines demonstrate that estrogen withdrawal can lead to PgR expression loss [100].

The decline in PgR expression is also promoted in a time-dependent manner by treatment with fulvestrant, as demonstrated in sequential biopsies of advanced BC [94]. Fulvestrant and the other SERDs have no agonistic activity and inhibit ligand binding to ERα, promote its degradation, and diminish transcription of ERα-dependent genes, including *PGR* [101]. Fulvestrant response rate seems independent from the baseline HER2 and PgR status because it antagonizes nuclear, cytoplasmatic, and membrane-bound ERs, completely inhibiting the cross-talk between the growth factor receptor and estrogen signaling [102]. Intriguingly, patients with a retained high PgR expression have a longer duration of response than patients with PgR loss at 6 weeks of treatment [101]. Moreover, overexpression of Tissue Inhibitor of Metalloproteinases-1 (TIMP1) ensues the down-regulation of PgR and drives resistance to fulvestrant in the MCF-7 cell line, but the mechanism of TIMP1-associated PgR depletion is unknown [103]. Resistance to fulvestrant may also be driven by mitogen-activated protein kinase (MAPK) pathway activation with increased levels of ERK, MEK, and RSK, kinases known to phosphorylate and inactivate PgR, hence, potentially, providing space for treatment with antiprogestins [104]. Phase 2 clinical trial investigating the combination of fulvestrant and onapristone for advanced or metastatic BC after progression on aromatase and CDK4/6 inhibitors (NCT04738292) is planned [105].

4. Genetic Landscape of ERα(+)/PgR(−) BC

Genomic alterations of ERα(+)/PgR(−) BC have been extensively studied in recent years. In terms of genetic stability, these tumors are characterized by increased DNA copy number gains when compared to double-positive BC cases [16]. Their mutation burden is intermediate between double-positive and triple-negative BCs [31]. In a comprehensive analysis of the large publicly available datasets, ERα(+)/PgR(−) tumors shared 5668 mutated genes with ERα(+)/PgR(+) cancers, while 1319 genes (19%) were uniquely altered in the former group [33]. The most commonly mutated genes were *PIK3CA*, *TP53*, *GATA3*, *CHD1*, *KMT2C*, *MUC16*, *MAP3K1*, *ARID1A*, *AHNAK2*, and *SYNE2* [29]. When compared to double-positive cancers, ERα(+)/PgR(−)/HER2(−) tumors displayed higher *TP53* and lower *PIK3CA* mutation rate, and more frequently showed amplification of oncogenes *ZNF703* and *RPS6KB1* [13,27].

Taking into consideration intrinsic molecular phenotypes, 15–46% of ERα(+)/PgR(−)/HER2(−) BCs are classified as PAM50-defined luminal A tumors, next 29–58% are classified as luminal B, and 20–27% as HER2-enriched or basal [31,106]. When compared to double-positive tumors, ERα(+)/PgR(−) BCs are characterized by lower endocrine sensitivity scores, enriched biosynthesis, metabolism, and DNA replication. The probability of benefits from ET in ERα(+)/PgR(−) tumors may be estimated also from three IHC markers: GATA3, CK5, and EGFR [31].

Analysis of mRNA expression profiles from several datasets demonstrated that ERα(+)/PgR(−) BCs share gene expression patterns both with double positive and double negative tumors [107]. This was confirmed also in our analysis of the TCGA dataset,

where we identified 2 and 32 differently expressed genes between ER(+)/PgR(−) and double-positive or double negative tumors, respectively. Importantly, we found only 10 genes uniquely differentiating between two subtypes of single hormone receptor-positive tumors [83].

5. The Biology of ERα(+)/PgR(−) BC

The biology of ERα(+)/PgR(−) BC cells is probably highly variable and depends on many cofactors (Figure 3). Isolated effects of ER (stimulated by estrogens) and PgR (stimulated by progestins) on gene expression are similar because they regulate the expression of shared target genes in the same direction (genomic agonism) [108]. In BC cells positive for both types of steroid hormone receptors, PgR competes with ERα regarding access to RNA polymerase III, and, hence, reduces its availability and ERα-dependent translation [84]. In consequence, when PgR expression is lost, ERα gains access to a broader range of translational machinery, which may promote tumor aggressiveness and growth. Moreover, chromatin binding of ERα is more consistent in double-positive tumors, whereas ERα binding patterns in PgR(−) subset are highly variable [108,109]. In PgR-deficient cells, ERα predominantly binds in the proximity to transcription start sites, whereas in PgR(+) cells PgR redirects ERα to bind distally to promoters. In consequence, in ERα(+)/PgR(−) BC ERα seem to act as a proximal promoter rather than distal enhancer of gene transcription, which stimulates pro-growth estrogenic signaling and reduces the responsiveness to ET [108]. Thus, PgR acts as a molecular rheostat regulating ER activity. Additionally, PgR mediates ERα chromatin binding to genes involved in cell death, apoptosis, and differentiation pathways and blocks ERα-dependent tumor growth [32]. Moreover, unliganded PgR regulates *ESR1* transcription via epigenetic modifications of the *ESR1* promoter. PgR depletion results in *ESR1* promoter hypermethylation, down-regulating expression of ER, which cannot be reversed after PgR re-expression [109].

The combined effect of estrogens and progestins on BC cells co-expressing ERα and PgR demonstrate that there is phenotypic antagonism between ERα and PgR. It has clinical consequences—in premenopausal patients, PgR has a more pronounced positive prognostic significance because of the availability of progesterone, which stimulates PgR signaling [110]. On the contrary, in post-menopausal females, progesterone levels are low, and thus are unable to produce a prominent phenotypic antagonism to ERα, which makes PgR expression a less important predictive factor in older patients.

Once PgR expression is lost, other receptors such as ERβ or androgen receptor (AR) may more significantly modulate ER-dependent actions. In the absence of PgR, AR most likely enhances ER-mediated transcription. In the nuclei of ER(+)/PgR(+) BC cells, AR competes with ER and PgR to bind to DNA, thus interfering with the estrogen-mediated transcription. Conversely, when PgR is lost, another receptor, ERβ, down-regulates ERα target genes, whereas AR enhances ERα target gene transcription and potentially contributes to tumor growth [111]. However, high AR expression is associated with prolonged relapse-free survival, lower grade, and lower number of affected lymph nodes in ERα(+)/PgR(−) BC, thus the mechanistic role of AR and its influence on ERα(+)/PgR(−) tumor aggressiveness requires further studies [112,113].

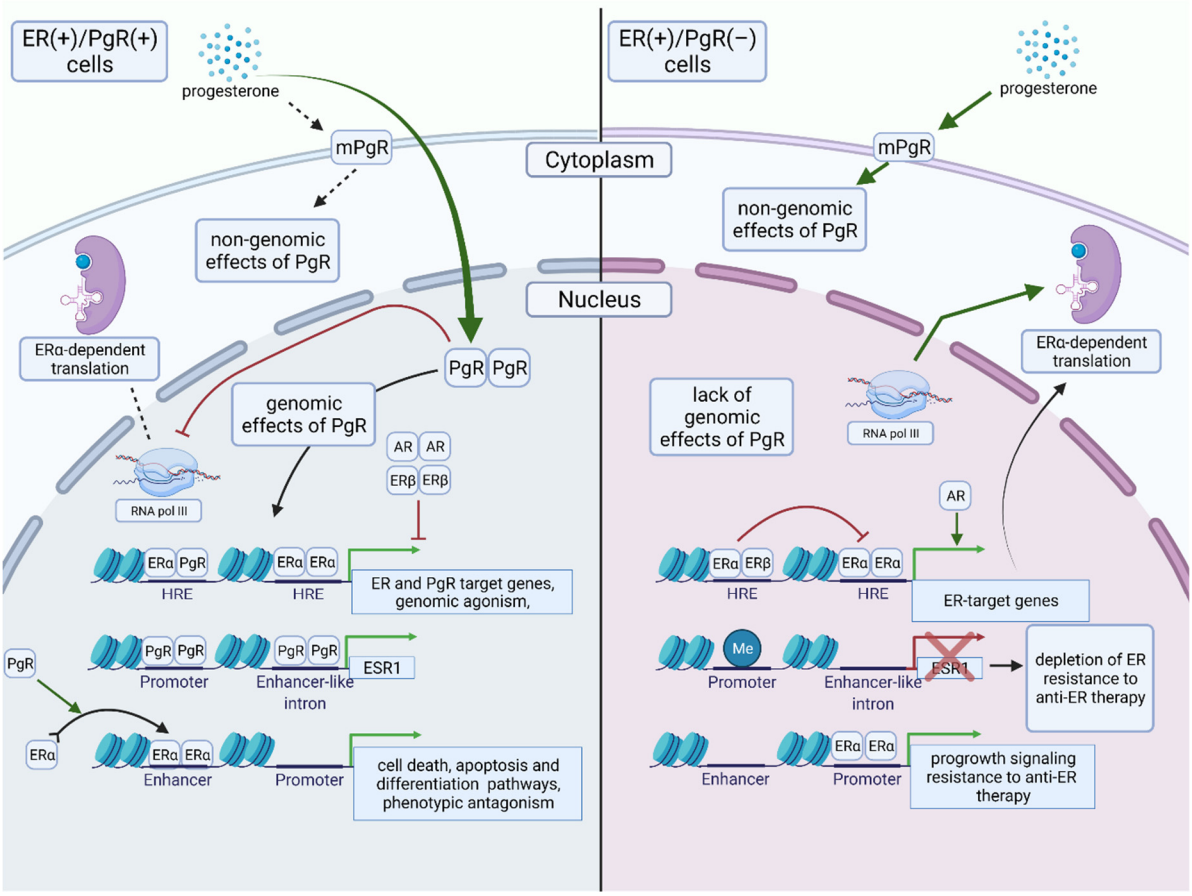

Figure 3. Biology of ERα(+)/PgR(+) and ERα(+)/PgR(−) breast cancer. Green arrows indicate stimulatory effects, red T-shaped lines depict inhibitory effects. In tumor cells co-expressing ERα and nuclear PgR the latter may exert both non-genomic and genomic effects. It regulates the expression of genes in a similar way to ERα (genomic agonism) but guides ERα binding to chromatin to induce expression of genes associated with good outcomes (phenotypic antagonism). PgR interacts with translational machinery (mainly RNA polymerase III) reducing its availability for ERα-dependent translation. Loss of nuclear PgR results in a shift of ERα role from distant enhancer to proximal promoter activating subset of genes associated with cancer progression. Depletion of PgR increases *ESR1* gene promoter methylation and down-regulates *ESR1*. Other steroid receptors, i.e., ER and AR may exert different effects on ERα-dependent genes expression in ERα(+)/PgR(+) and ERα(+)/PgR(−) breast cancers. For details, see text. Abbreviations: AR—androgen receptor; *ESR1*—estrogen receptor 1 gene; ER—estrogen receptor; HRE—hormone receptor element; (m)PgR—(membranous) progesterone receptor, RNA pol III—RNA polymerase III. Created with BioRender.com—accessed date 22 September 2021.

The loss of nuclear PgR expression does not imply loss of progestin responsiveness in BC cells [114]. Similarly to estrogens, progestins may act via membrane receptors (mPgRs), which have three subtypes: mPgRα, mPgRβ, and mPgRγ, the first being the most prevalent in breast tissue [115]. In PgR(−) BC cell lines progesterone produces an antiapoptotic response and activates MAPK and PI3K/Akt through mPgRs [114,116]. Expression of mPgR was correlated with HER2-overexpression, a number of lymph node metastases, and a worse prognosis in BC [117]. Thus, mPgRs might be important players in the biology of ERα(+)/PgR(−) BCs providing pro-growth signals. Nevertheless, some in vitro studies utilizing BC cell lines demonstrated that mPgRα mediates antiproliferative and antimetastatic signaling of progesterone [118,119], although the effects of mPgRs are potentially dependent on the model (in vitro vs. in vivo or clinical studies), progesterone levels, and competition with nuclear receptors. Of note, there is an inverse relationship between nuclear PgR and mPgR [117].

A recent study in PgR-low/null tumors defined phospho-PgR target gene sets (*ERBB2*, *PAX2*, *AHR*, *AR*, and *RUNX2*) which regulate cancer stem cell biology and increase tumor heterogeneity [85]. Paradoxically, antiprogestin treatment may possibly be effective in these clinically PgR(−) tumors, preventing the development of endocrine resistance [85]. However, not all antiprogestins are equally adequate to this approach, since it was shown that in the presence of progesterone onapristone blocks Ser294 phosphorylation, whereas mifepristone and aglepristone induce Ser294 phosphorylation, behaving similar to partial agonists of PgR [85]. Phase I study of onapristone in heavily pre-treated, metastatic endometrial, ovarian, and BC showed promising results and proposed activated progesterone receptor as a potential predictive factor [120].

The understanding of PgR significance in BC is further complicated by the coexistence of its isoforms, as phosphorylated PgR-A is a more potent driver of cancer stem cell expansion, whereas PgR-B is involved in BC cells proliferation [121]. In normal mammary gland tissue, the levels of PgR-A and PgR-B are similar, while the ratio is disturbed during cancer transformation, usually resulting in PgR-A prevalence [122]. In vitro studies demonstrated that the PgR-A/PgR-B ratio determines the functional outcome of PgR action, including both the target genes and response to hormones and growth factors [123]. This observation was further confirmed in clinics because a high PgR-A/PgR-B ratio was indicative of a shorter time to relapse in patients treated with tamoxifen within the ATAC trial [124]. Interestingly, it is speculated that tamoxifen resistance and the worse prognosis are associated solely with methylation of *PGRA* promoter, resulting in the functional predominance of PgR-B [57]. High frequency of ERα:PgR-B interaction was predictive of relapse on an adjuvant AI, and in some cases, a substantial amount of ERα:PgR-B interactions coexisted with a lack of IHC-detectable PgR expression [125].

It was recently shown that among HER2-negative tumors ERα(+)/PgR(−) BCs display distinctive tyrosine kinases profiles [126], characterized by higher overall kinase activity than double-positive tumors, with RAS, PI3K, and ErbB signaling being mostly responsible for these differences. Four kinases showed significant expression differences between PgR(−) and PgR(+) tumors: fibroblast growth factor receptor 4 (FGFR4) and LCK were up-regulated, whereas Fyn-related kinase (FRK) and macrophage-stimulating 1 receptor (MST1R) down-regulated in PgR(−) cases. Interestingly, all these kinases are directly regulated by progesterone. Moreover, Tahiri et al. identified 24 kinase-encoding genes differentially expressed between double-positive and PgR(−) tumors, dividing ER(+)/HER2(−) BCs into two prognostically distinct clusters: cluster 1 comprising mostly PgR(+) patients with a better prognosis, and cluster 2 characterized by worse prognosis and the predominance of PgR(−) patients [126]. Additionally, PgR(−) patients in cluster 2 had inferior survival to PgR(−) patients in cluster 1. Unfortunately, the association between the clusters and luminal A vs. B phenotype was not studied. Importantly, these associations are not seen in HER2(+) samples, suggesting that the effects of HER2 are dominant. This is further supported by our study on single hormone receptor BC, in which miRNA profiles of single hormone receptor-positive breast cancers were mainly dependent on the status of HER2, rather than on ERα/PgR status [83].

6. Conclusions

Lack of PgR expression in ERα(+) BC has multiple potential explanations but the molecular, pathological and clinical heterogeneity of this group remains underappreciated. The biology of ERα(+)/PgR(−) BC is context-dependent, being highly modulated by the cross-talk between growth factors receptors and nuclear or membranous steroid hormone receptors. Novel therapeutic targets as microRNAs, epigenetic modifications, tyrosine kinases, and transcriptionally overactive PgR should be further investigated in the future. Identification of the mechanism of PgR loss in each patient seems challenging, yet it may provide important information on the biology of the tumor and predict its responsiveness to the therapy. Finally, future studies should focus on the investigation of novel biomarkers

predicting the disease course, as well as its response to endocrine and chemotherapy in this distinctive group of patients.

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microRNA Expression Profile in Single Hormone Receptor-Positive Breast Cancers Is Mainly Dependent on HER2 Status—A Pilot Study

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Abstract: Estrogen (ER) and progesterone (PgR) receptors and HER2 are crucial in the assessment of breast cancer specimens due to their prognostic and predictive significance. Single hormone receptor-positive breast cancers are less common and their clinical course is less favorable than ER(+)/PgR(+) tumors. Their molecular features, especially microRNA (miRNA) profiles, have not been investigated to date. Tumor specimens from 36 chemonaive breast cancer patients with known ER and PgR status (18 ER(+)/PgR(−) and 18 ER(−)/PgR(+) cases) were enrolled to the study. The expression of 829 miRNAs was evaluated with nCounter Human v3 miRNA expression Assay (NanoString). miRNAs differentiating between ER/PgR/HER2 phenotypes were selected based on fold change (FC) calculated for the mean normalized counts of each probe in compared groups. The differences were estimated with Student's *t*-test or Two-Way ANOVA (considering also the HER2 status). The results were validated using The Cancer Genome Atlas (TCGA) dataset. Following quality control of raw data, fourcases were excluded due to low sample quality, leaving 14 ER(+)/PgR(−) and 18 ER(−)/PgR(+) cases. After correction for multiple comparisons, we did not find miRNA signature differentiating between ER(−)/PgR(+) and ER(+)/PgR(−) breast cancers. However, a trend for differing

expression (p-value ≤ 0.05; FDR > 0.2; ANOVA) in eight miRNAs was observed. The ER(+)/PgR(−) group demonstrated elevated levels of four miRNAs—miR-30a-5p, miR-29c-3p, miR-141-3p and miR-423-5p—while the ER(−)/PgR(+) tumors were enriched in another four miRNAs—miR-514b-5p, miR-424-5p, miR-495-3p, and miR-92a-3p. For one of the miRNAs—miR-29c-3p—the association with the ER(+)/PgR(−) phenotype was confirmed in the TCGA cohort (p-value = 0.024; t-test). HER2 amplification/overexpression in the NanoString cohort was related to significant differences observed in 33 miRNA expression levels (FDR ≤ 0.2; ANOVA). The association with HER2 status was confirmed in the TCGA cohort for four miRNAs (miR-1180-3p, miR-223-3p, miR-30d-5p, and miR-195-5p). The main differences in miRNA expression amongst single hormone receptor-positive tumors were identified according to their HER2 status. However, ER(+)/PgR(−) cases tended to express higher levels of miRNAs associated with ER-positivity (miR-30a-5p, miR-29c-3p, miR-141-3p), whereas ER(−)/PgR(+) cancers showed elevated levels of miRNAs characteristic for double- and triple-negative tumors (miR-92a-3p, miR-424-5p). Further studies are necessary to comprehensively analyze miRNA signatures characteristic of ER(−)/PgR(+) and ER(+)/PgR(−) tumors.

Keywords: breast cancer; estrogen receptor; progesterone receptor; HER2; microRNA

1. Introduction

Breast cancer is the most frequent malignancy and the most common cause of cancer-related death in women worldwide. The expression of estrogen receptor (ER), progesterone receptor (PgR), and HER2 are crucial in the assessment of breast cancer specimens due to their prognostic and predictive significance. PgR expression in the mammary gland is dependent on ER, thus, these two receptors are usually co-expressed [1]. In 15% of cases, PgR expression is lost in ER(+) cancers, whereas a lack of nuclear ER expression in PgR(+) tumors is unusual. ER(+)/PgR(−) tumors tend to present less favorable clinicopathological features and a higher risk of relapse than ER+/PgR+ cancers [2,3]. Loss of PgR expression may be related to various mechanisms, including nonfunctional ER, epigenetic modifications of PgR promoter, low levels of circulating estrogens, and altered ER co-regulators [4]. The existence of ER(−)/PgR(+) tumors has been questioned and mostly regarded as an artifact in immunohistochemical staining. Nevertheless, they are still encountered in practice; our own experience and thorough literature analysis indicate that at least some cases of ER(−)/PgR(+) tumors are non-artifactual [1,5] but are characterized by a unique clinical course and biological features, including high-grade histology and the prognosis being an intermediate between triple-negative and double-positive tumors.

MicroRNAs (miRNAs) are short non-coding RNAs, which regulate gene expression. They are transcribed by RNA polymerase II. Precursor forms of miRNAs are processed by endoribonuclease Dicer in the cytoplasm. Subsequently, they are incorporated into RNA-induced silencing complex (RISC) and modulate expression of genes via mRNA cleavage and degradation or translational repression [6].

miRNAs have multiple roles in cancer biology as they may serve as tumor suppressors and oncogenes (tsmiRs and oncomiRs, respectively) [7]. Nevertheless, their significance is much broader, since they regulate cancer cell metabolism and host immune response and expression of potentially targetable proteins [8]. In neoplastic cells, some miRNAs are upregulated, whereas others are downregulated, thus their differential expression may potentially serve as diagnostic, prognostic, and predictive markers in various malignancies, including breast cancer [9].

miRNA expression profile of single hormone receptor-positive tumors has been poorly investigated so far. However, studies focused on ER+/PgR+ breast cancer indicate that miRNAs interact reciprocally with ER and PgR receptors [10]. Recently, small RNA sequencing of 186 tumor samples showed that miRNA expression can be translated into intrinsic molecular subtypes of breast cancer [11].

The cluster consisting of miR-99a/let-7c/miR-125b miRNA separated luminal A and luminal B subtypes, whereas miR-4728 was a specific marker of the HER2-enriched subgroup.

In the current pilot study we aimed to identify differentially expressed miRNAs in two types of single hormone receptor-positive breast cancers (ER(+)/PgR(−) and ER(−)/PgR(+)) with further distinction into HER2-overexpressing/amplified and HER2-negative tumors in a well-established cohort collected at the Medical University of Gdańsk. Owing to the complexity of breast cancer and the essential role of both HER2 and hormone receptors (ER and PgR) in its biology, we were interested in evaluating the differences in miRNA profiles between the four ER/PgR/HER2 phenotypes of single hormone receptor-positive primary breast tumors. To validate the results we used a publicly available dataset from The Cancer Genome Atlas Project (TCGA, https://www.cancer.gov/tcga).

2. Materials and Methods

2.1. Study Group

A total of 96 breast cancer patients diagnosed with a confirmed single hormone receptor-positive tumor (64 ER(+)/PgR(−) and 32 ER(−)/PgR(+)) were screened for eligibility. The patients were diagnosed in 9 Polish centers (Medical University of Gdańsk; Lower Silesian Oncology Center, Wrocław; Tadeusz Koszarowski Regional Oncology Center, Opole; Medical University of Łódź; Gdynia Maritime Hospital; Greater Poland Cancer Centre, Poznań; The Maria Skłodowska-Curie National Research Institute of Oncology, Warsaw; Beskid Oncology Center, Bielsko-Biała; Copernicus Hospital, Gdańsk) between 2007 and 2018.

All cases underwent central review by pathologists experienced in breast cancer (R.P. and M.K.) to confirm the diagnosis and receptor status. Three antibodies against ER were utilized (Dako monoclonal (MC) mouse anti-ERα, clone 1D5; Dako MC rabbit anti-ERα, clone EP1; VENTANA Roche MC rabbit anti-ERα, clone SP1), and one against PgR (Dako MC mouse anti-PgR, clone 636). Only cases with < 1% stained tumor nuclei were regarded as negative for a given receptor according to American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) criteria (Hammond et al. 2010). Histologically normal breast epithelium adjacent to carcinoma was used as an internal positive control. HER2 status was routinely evaluated by immunohistochemistry and/or by hybridization in situ and was obtained from the medical records. Subsequently, propensity score matching was performed using the Matching package [12] according to age, grade, HER2 status, and Ki67 status. Only cases with a sufficient amount of tumor tissue for molecular testing were enrolled. Thus, 36 paired single hormone receptor-positive cases were included (18 ER(+)/PgR(−) and 18 ER(−)/PgR(+)). The study was approved by the Bioethical Committee of the coordinating center, Medical University of Gdansk, Poland (approval no: NKBBN/119/2018; 10 April 2018). All research was performed in accordance with the appropriate regulations.

2.2. NanoString nCounter Assay for miRNA Profiling

Total RNA, including miRNA, was isolated from archival FFPE blocks (four 20 µm-thick sections per block) using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Invitrogen, Carlsbad, CA, USA) following the manufacturers' protocol. RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Extracted RNA (3 µl) was subjected to miRNA expression profiling with nCounter Human v3 miRNA Expression Assay (NanoString Technologies, Seattle, WA, USA) according to the manufacturer's procedures for hybridization, detection, and scanning [13]. Raw NanoString expression data were submitted to the GEO database under GSE155362 accession number.

Following quality control of raw data, 4 cases were excluded from analysis due to low sample quality and resulting ligation issues, thus the final study group counted 14 ER(+)/PgR(−) and 18 ER(−)/PgR(+) cases (characterised in Table 1).

Table 1. Clinicopathological characteristics of patients with single hormone receptor-positive breast tumors in the NanoString cohort; differences estimated with *t*-test (age, Ki67, tumor size) or Fisher's Exact test (grade, estrogen receptor (ER) status, progesterone receptor (PgR) status, human epidermal growth factor receptor 2 (HER2) status, T, N, M); significant results (p-value < 0.05) are in bold.

Parameter		All (n = 32)		ER(+)PgR(−) (n = 14)		ER(−)PgR(+) (n = 18)		p-Value
Age	median (range)	62	29–78	66	36–76	53.5	29–78	0.141
Grade	1	0	0%	0	0%	0	0%	**0.003**
	2	6	19%	6	19%	0	0%	
	3	26	81%	8	25%	18	56%	
ER status	negative	18	56%	0	0%	18	56%	**<0.001**
	positive	14	44%	14	44%	0	0%	
PgR status	negative	14	44%	14	44%	0	0%	**<0.001**
	positive	18	56%	0	0%	18	56%	
HER2 status	negative	15	47%	5	16%	10	31%	0.308
	positive	17	53%	9	28%	8	25%	
Ki67	median (range)	47.5	9–90	30	9–70	60	30–90	**<0.001**
Tumor size [mm]	median (range)	21	8–47	21.5	12–30	21	8–47	0.216
T	1	14	44%	6	19%	8	25%	0.963
	2	13	41%	6	19%	7	22%	
	3	1	3%	1	3%	0	0%	
	4	3	9%	1	3%	2	6%	
	NA	1	3%	0	0%	1	3%	
N	0	17	53%	8	25%	9	28%	0.351
	1	11	34%	6	19%	5	16%	
	2	3	9%	0	0%	3	9%	
	NA	1	3%	0	0%	1	3%	
M	0	29	91%	13	41%	16	50%	1.000
	1	2	6%	1	3%	1	3%	
	NA	1	3%	0	0%	1	3%	

For each analyzed sample, correction and normalization were performed using nSolver 4.0 software, as previously described [14]. 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 all miRNA genes. The negative and positive control probes were included in the assay.

Transcripts detected in <1/3 of the whole NanoString group (<10 cases) were excluded, leaving 185 out of 798 miRNAs for further analysis.

2.3. The Cancer Genome Atlas (TCGA) miRNA Data Processing

Clinical and miRNA-seq data of the Breast invasive carcinoma (BRCA) cohort were obtained from the TCGA portal (data status of 28 January 2016). The methods of biospecimen procurement, RNA isolation, and RNA sequencing were previously described by The Cancer Genome Atlas Research Network [15,16].

The Illumina HiSeq miRNA-seq dataset (illuminahiseq_mirnaseq-miR_gene_expression), covering normalized counts of sequences aligning to 1046 miRNA transcripts ("reads_per_million_miRNA_mapped") in 756 primary breast tumors, were selected for analysis. Records with missing clinical or expression values were excluded. The group was limited to

single hormone receptor-positive tumors—ER(+)/PgR(−) and ER(−)/PgR(+)—from female patients, not exposed to neoadjuvant systemic therapy, with known HER2 status, leaving 67 out of 756 cases for further analysis (characterized in Table 2 and listed in Supplementary Table S1). The overlap between our series (further referred to also as the NanoString group; miRBase version 21) and the TCGA dataset (miRBase version 16) was determined using the miRBaseConverter package [17]. Two hundred and twelve (212) miRNAs were assessed in both NanoString and TCGA data. Due to the unbalanced proportions of ER/PgR/HER2 subgroups in the TCGA cohort (reflecting the population frequency), the power of the analysis was limited, which precluded the screening of all miRNAs. Thus, only the miRNAs differing between subgroups in the NanoString data were investigated in the TCGA dataset (the chart of data processing is shown in Supplementary Figure S1 and a list of miRNA analysis is given in Supplementary Table S2).

Table 2. Clinicopathological characteristics of patients with single hormone receptor-positive breast tumors in a TCGA breast invasive carcinoma cohort; differences estimated with *t*-test (age, Ki67, tumor size) or Fisher's Exact test (ER status, PgR status, HER2 status, T, N, M); significant results (p-value < 0.05) are in bold.

Parameter		All (n = 67)		ER(+)PgR(−) (n = 57)		ER(−)PgR(+) (n = 10)		p-Value
Age	median (range)	60	30–90	61	30–90	55.5	46–90	0.486
ER status	negative	10	15%	0	0%	10	15%	**<0.001**
	positive	57	85%	57	85%	0	0%	
PgR status	negative	57	85%	57	85%	0	0%	**<0.001**
	positive	10	15%	0	0%	10	15%	
HER2 status	negative	53	79%	46	69%	7	10%	0.425
	positive	14	21%	11	16%	3	4%	
T	1	13	19%	12	18%	1	1%	0.437
	2	44	66%	35	52%	9	13%	
	3	9	13%	9	13%	0	0%	
	4	1	1%	1	1%	0	0%	
	NA	0	0%	0	0%	0	0%	
N	0	35	52%	25	37%	10	15%	**0.028**
	1	22	33%	22	33%	0	0%	
	2	3	4%	3	4%	0	0%	
	3	5	7%	5	7%	0	0%	
	NA	2	3%	2	3%	0	0%	
M	0	48	72%	39	58%	9	13%	0.260
	1	0	0%	0	0%	0	0%	
	NA	19	28%	18	27%	1	1%	

2.4. miRNA Targets Prediction and Functional Annotation

miRNET 2.0 database including miRTarBase 8.0 (www.mirnet.ca) was employed to identify target genes of selected miRNAs in mammary gland tissue [18]. The experimentally confirmed targets were subjected to functional annotation analysis (Gene Ontology biological processes (GO BP) using the Functional Annotation Tool by DAVID Bioinformatics Resources 6.81) [19,20].

2.5. The Cancer Genome Atlas (TCGA) mRNA Data Processing

mRNA-seq data (RNASeqV2, RSEM_ normalized), covering normalized counts of sequences aligning to 20,531 mRNA transcripts in 1091 primary breast tumors, were obtained from the TCGA

portal (data status of 28 January 2016). Records with missing clinical or expression values were excluded. The group was limited to tumors with known hormone receptor status (ER and PgR) from female patients not exposed to neoadjuvant systemic therapy, leaving 1012 out of 1091 cases for further analysis (listed in Supplementary Table S1). The distribution of ER/PgR phenotypes in the group was as follows: ER(−)/PgR(-)—218 (22%), ER(−)/PgR(+)—16 (2%), ER(+)/PgR(−)—117 (12%), ER(+)/PgR(+)—661 (65%).

Analysis of reciprocal miRNA-mRNA expression was performed on the Illumina HiSeq sub-cohort of single hormone receptor-positive patients (n = 67) with miRNA profiling data available. For each of the top20 GO BP terms enriched in miRNA targets identified in the NanoString cohort, mRNA targets of ER/PgR-associated miRNAs (5/8 available in TCGA dataset) were extracted and their expression was correlated with the targeting miRNAs. For GO BP terms with at least 5 mRNA targets for each miRNA, the overlap of correlated mRNAs (cor > 0.3 or cor ≤ −0.3; Pearson's method) between miRNAs was illustrated with Venn diagrams [21].

Analysis of unique mRNA-differentiating single hormone receptor-positive tumors from other phenotypes, or the single hormone receptor-positive tumors from each other (ER(+)/PgR(−) vs ER(−)/PgR(+)), was performed on the whole TCGA mRNA-seq cohort (n = 1012). For each mRNA transcript, differences in expression between compared phenotypes were reported as log2FC and estimated using t-test with Benjamini–Hochberg correction for multiple testing. Genes with log2FC > 1 or log2FC ≤ 1 and FDR > 0.05 were classified as differentiating between the compared phenotypes. Unique differentiating genes were identified via Venn diagram-based analysis of overlap between the lists generated for all compared groups.

3. Statistical Analysis

The data were analyzed using the R statistical environment (3.6.1) [22]. miRNAs differentiating between ER/PgR/HER2 phenotypes were selected based on logarithmic fold change (log2FC) calculated for the mean normalized counts of each probe in compared groups. miRNAs with log2FC ≥ 0.3 were considered upregulated; miRNAs with log2FC < −0.3 were considered downregulated. The differences were estimated with Student's t-test (for ER/PgR and HER2(+)/HER2(−) comparisons) or Two-Way ANOVA (for ER/PgR/HER2 comparisons) with Benjamini–Hochberg correction for multiple testing. Differences in distribution of categorical variables between groups (clinicopathological characteristics) were estimated using Fisher's Exact test. Correlation between linear variables (miRNA and mRNA expression) was estimated using Pearson's method. p-values ≤ 0.05 and false discovery rate (FDR) values ≤ 0.2 were considered statistically significant.

Propensity score matching for ER(+)/PgR(−) and ER(−)/PgR(+) groups was performed using the Matching package [12]. The overlap between NanoString dataset (miRBase version 21) and the TCGA dataset (miRBase version 16) was determined using the miRBaseConverter package [17]. Heatmap was generated using heatmap3 package [18] and Venn diagrams were generated using venn package [21].

4. Results

4.1. Comparison of Study Groups

ER(−)/PgR(+) tumors were characterized by a higher grade and a higher Ki-67 index than ER(+)/PgR(−) cancers in the NanoString cohort, whereas in the TCGA cohort, ER(+)/PgR(−) patients presented more frequently with positive lymph nodes (Tables 1 and 2). When compared to the TCGA cohort, our group was overrepresented by T1 tumors, and HER2-overexpressing/amplified cases (Supplementary Figure S2).

4.2. miRNA Expression Profile Associated with HER2 Status

HER2 amplification/overexpression was related to significant differences observed in 33 miRNA expression levels (FDR ≤ 0.2; ANOVA). Eleven miRNAs were overexpressed and 22 miRNAs were

under-expressed in HER2-positive cancers when compared to HER2-negative cancers (Table 3, Supplementary Figure S3). The most upregulated was miR-887-5p (FC 7.21), while miR-660-5p was the most downregulated (FC 0.20). Differentially expressed miRNAs are represented with a heatmap visualization (Figure 1), and with a volcano plot (Supplementary Figure S4).

Table 3. HER2-associated miRNAs in single hormone receptor-positive breast tumors; log2fold change (log2FC) calculated for the mean normalized counts of each probe in compared groups—HER2(+) vs. HER2(−); miRNAs upregulated in HER2(+) tumors are marked with ↑, miRNAs downregulated in HER2(+) tumors are marked with ↓; differences estimated with Two-Way ANOVA (*p*-value) with Benjamini–Hochberg correction (FDR), only statistically significant results (FDR ≤ 0.2) are presented; miRNA names are according to miRBase database (v21).

miRNA	HER2 log2FC	Direction	HER2 *p*-Value	HER2 FDR	ER/PgR log2FC	ER/PgR *p*-Value	ER/PgR FDR
hsa-miR-887-5p	2.85	↑	0.003	0.130	0.42	0.990	0.998
hsa-miR-208a-3p	2.50	↑	0.012	0.156	0.79	0.541	0.847
hsa-miR-891a-5p	2.29	↑	0.020	0.160	0.85	0.473	0.826
hsa-miR-301a-5p	2.17	↑	0.026	0.160	1.26	0.178	0.811
hsa-miR-33b-5p	2.09	↑	0.024	0.160	0.69	0.598	0.864
hsa-miR-1296-3p	1.91	↑	0.007	0.130	0.53	0.675	0.898
hsa-miR-378d	1.91	↑	0.002	0.130	0.14	0.702	0.908
hsa-miR-548v	1.82	↑	0.027	0.160	0.82	0.400	0.811
hsa-miR-1295a	1.73	↑	0.005	0.130	0.73	0.338	0.811
hsa-miR-337-3p	1.54	↑	0.014	0.156	−0.11	0.480	0.827
hsa-miR-514b-5p	0.83	↑	0.023	0.160	−0.67	0.046	0.809
hsa-miR-185-5p	−0.71	↓	0.032	0.186	0.13	0.410	0.811
hsa-miR-340-5p	−0.72	↓	0.025	0.160	−0.27	0.666	0.898
hsa-miR-424-5p	−0.74	↓	0.020	0.160	−0.69	0.048	0.809
hsa-miR-106a-5p+hsa-miR-17-5p	−0.82	↓	0.007	0.130	−0.40	0.379	0.811
hsa-miR-151a-3p	−0.84	↓	0.021	0.160	0.29	0.221	0.811
hsa-miR-374a-5p	−0.91	↓	0.013	0.156	0.10	0.435	0.811
hsa-miR-141-3p	−0.92	↓	0.034	0.193	1.10	0.018	0.809
hsa-miR-26b-5p	−0.96	↓	0.025	0.160	0.54	0.110	0.809
hsa-miR-126-3p	−0.97	↓	0.006	0.130	0.20	0.255	0.811
hsa-miR-32-5p	−1.04	↓	0.017	0.160	0.19	0.349	0.811
hsa-miR-15b-5p	−1.08	↓	0.023	0.160	−0.55	0.425	0.811
hsa-miR-30d-5p	−1.12	↓	0.006	0.130	0.53	0.078	0.809
hsa-miR-1180-3p	−1.18	↓	0.008	0.142	−0.16	0.867	0.953
hsa-miR-30b-5p	−1.25	↓	0.002	0.130	0.13	0.310	0.811
hsa-miR-195-5p	−1.33	↓	0.024	0.160	0.49	0.209	0.811
hsa-miR-429	−1.39	↓	0.023	0.160	−0.27	0.997	0.998
hsa-miR-503-5p	−1.46	↓	0.011	0.156	−1.19	0.079	0.809
hsa-miR-223-3p	−1.47	↓	0.025	0.160	−1.30	0.087	0.809
hsa-miR-450a-5p	−1.60	↓	0.006	0.130	−0.74	0.397	0.811
hsa-miR-29b-3p	−1.63	↓	0.001	0.130	0.36	0.135	0.811
hsa-miR-135b-5p	−2.17	↓	0.016	0.160	−1.46	0.184	0.811
hsa-miR-660-5p	−2.30	↓	0.010	0.147	−1.49	0.167	0.811

These results were partially validated in the TCGA cohort (23/33 available for analysis), confirming HER2-related downregulation of four miRNAs from our cohort (miR-30d-5p, miR-1180-3p, miR-195-5p, and miR-223-3p) in the TCGA dataset (FDR ≤ 0.2; *t*-test).

Functional analysis of gene ontology revealed that the predicted gene targets (3925; Supplementary Table S3) of HER2-associated miRNAs are mostly involved in transcription regulation, but also in cellular matrix organization, regulation of cell cycle or apoptosis; apart from cancer-associated pathways, the most altered signaling pathways included PI3-K-Akt, p53, and FoxO (Figure 2, Supplementary Table S4).

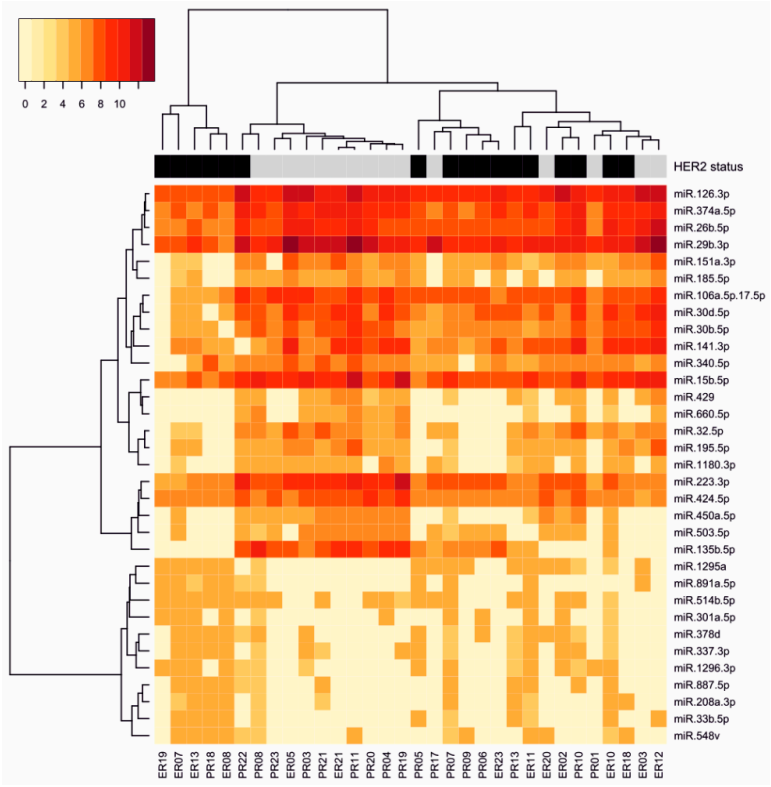

Figure 1. Heatmap depicting expression of 33 HER2-associated miRNAs among analyzed single hormone receptor-positive breast tumors; color legend indicates HER2 status; HER2-negative cases are marked in grey, HER2-positive cases are marked in black.

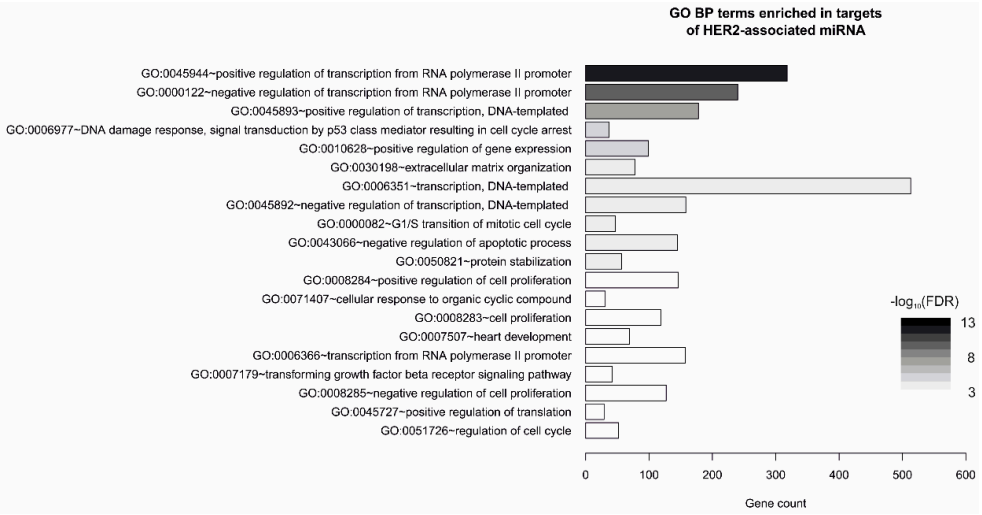

Figure 2. Gene Ontology Biological Process (GO BP) terms enriched in genes targeted by HER2-associated miRNAs in single hormone receptor-positive breast tumors; top20 terms/pathways with the lowest *p*-value plotted as the number of associated genes (gene count) and ordered according to -log 10(FDR); analyzed with Functional Annotation Tool by DAVID Bioinformatics Resources 6.81.

4.3. miRNAs Associated with Steroid Hormone Receptor Expression

In the NanoString cohort we did not identify any miRNA significantly different between both single hormone receptor-positive subgroups of breast cancer. Nevertheless, we observed a trend for differing expression (p-value ≤ 0.05; FDR > 0.2; ANOVA) in eight miRNAs. ER(+)/PgR(−) group demonstrated elevated levels of four miRNAs—miR-30a-5p, miR-29c-3p, miR-141-3p, and miR-423-5p—while the ER(−)/PgR(+) tumors were enriched in another four miRNAs—miR-514b-5p, miR-424-5p, miR-495-3p, miR-92a-3p (Table 4, Supplementary Figure S5). For one of the miRNAs—miR-29c-3p—the association with the ER(+)/PgR(−) phenotype was confirmed in the TCGA cohort (p-value = 0.024; t-test). Volcano plot of ER/PgR-associated miRNAs in single hormone receptor-positive breast tumors is shown in Supplementary Figure S6.

Table 4. ER/PgR-associated miRNAs in single hormone receptor-positive breast cancers; log2fold change (log2FC) calculated for the mean normalized counts of each probe in compared groups—ER(+)/PgR(−) vs. ER(−)/PgR(+); miRNAs upregulated in ER(+)/PgR(−) tumors are marked with ↑, miRNAs downregulated in ER(+)/PgR(−) tumors are marked with ↓; differences estimated with Two-Way ANOVA (p-value) with Benjamini–Hochberg correction (FDR), only statistically significant results (p-value ≤ 0.05) are presented; miRNA names according to the miRBase database (v21).

miRNA	ER/PgR log2FC	Direction	ER/PgR *p*-Value	ER/PgR FDR	HER2 log2FC	HER2 *p*-Value	HER2 FDR
hsa-miR-30a-5p	1.91	↑	0.031	0.809	−1.69	0.046	0.221
hsa-miR-29c-3p	1.40	↑	0.030	0.809	−1.23	0.047	0.221
hsa-miR-141-3p	1.10	↑	0.018	0.809	−0.92	0.034	0.193
hsa-miR-423-5p	0.73	↑	0.045	0.809	−0.49	0.119	0.338
hsa-miR-514b-5p	−0.67	↓	0.046	0.809	0.83	0.023	0.160
hsa-miR-424-5p	−0.69	↓	0.048	0.809	−0.74	0.020	0.160
hsa-miR-495-3p	−2.05	↓	0.027	0.809	−0.51	0.773	0.851
hsa-miR-92a-3p	−2.32	↓	0.033	0.809	−1.27	0.206	0.419

Gene targets of the miRNAs potentially associated with the single hormone receptor-positive phenotype (3011; Supplemantary Table S3), were subjected to enrichment analysis. The identified pathways were mostly involved in cell–cell adhesion, as well as regulation of transcription, cell cycle and cell division (Figure 3, Supplementary Table S5). To further explore the association between selected miRNAs, their targets and enriched pathways, mRNA targets of ER/PgR-associated miRNAs (5/8 available in TCGA dataset) were matched with genes associated with a given GO BP term and the expression of each miRNA was correlated with expression of its mRNA targets. For each GO BP term, the overlap between mRNA correlating with miRNA of interest was illustrated with a Venn diagram (Supplementary Figure S7). The most significant effect (the number of selected miRNA targets involved in each pathway) was observed for miR-29c-3p and miR-141-3p.

Additionally, we analyzed mRNA-differentiating ER/PgR phenotypes based on the TCGA dataset, and found 10 genes uniquely differentiating between two subtypes of single hormone receptor-positive breast cancer (Supplementary Figure S8). Correlation between expression of ER/PgR-associated miRNA and targeted mRNA was estimated for four available miRNA-mRNA (*TGFB2*–miR-141-3p, *NEDD4L*–miR-30a-5p, *FGFR4*–miR-424-5p, *SOCS2*–miR-424-5p) was assessed, but no significant results were obtained (Supplementary Figure S9).

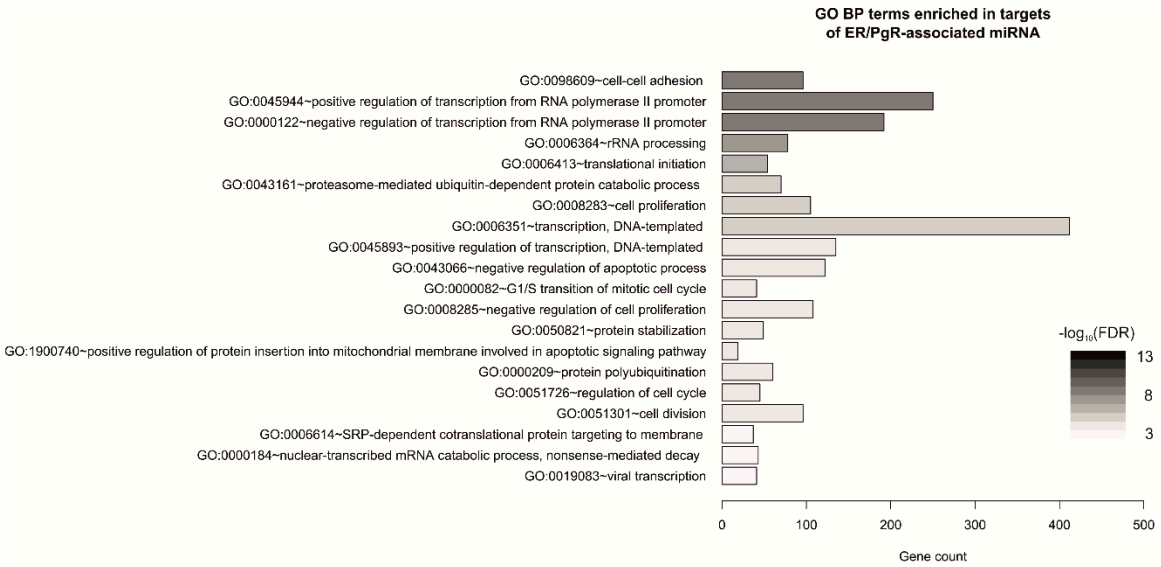

Figure 3. Gene Ontology Biological Process (GO BP) terms enriched in genes targeted by ER/PgR-associated miRNAs in single hormone receptor-positive breast cancers; top20 terms/pathways with the lowest *p*-value plotted as the number of associated genes (gene count) and ordered according to -log 10(FDR); analyzed with Functional Annotation Tool by DAVID Bioinformatics Resources 6.81.

5. Discussion

In this pilot study of miRNA expression profiling in single hormone receptor-positive breast cancer we demonstrated that miRNA expression profiles of these tumors depend mainly on their HER2 status, rather than on their hormonal receptor status. However, we also found several candidate miRNAs which could be potentially associated with either an ER(−)/PgR(+) or an ER(+)/PgR(−) subtype of breast cancer, which may indicate their biological importance in these tumors.

Four miRNAs in our study showed a decreased expression in HER2-overexpressing/amplified tumors in both NanoString and TCGA cohorts. Three of them (miR-30d-5p, miR-195-5p, and miR-223-3p) were previously reported to be downregulated in HER2-overexpressing/amplified cancers [22–24]. miR-223-3p is also downregulated in HER2-overexpressing C5.2 cell line [25]. Citron et al. postulated a central role for miR-223 in the control of epidermal growth factor signaling and HER2 activation, as it reduces the oncogenic potential of HER2-transformed mammary epithelial cells [26]. Moreover, activation of HER2 downregulates miR-223-3p via RB repression and E2F1 activation [26]. Another miRNA—miR-30d—was upregulated by trastuzumab in BT474 cells [27]. In ovarian carcinoma, a lower expression of the miR-30 family, including miR-30d, was associated with HER2 overexpression [23]. Two miRNAs identified in our cohort—miR-30d and mir-195-5p—inhibit the cell cycle by targeting cyclin E [28,29]. Consistently, their lower expression was noted in biologically aggressive types of breast cancer, i.e., HER2-enriched and basal-like carcinomas [30]. As expected, G1/S transition of mitotic cycle and regulation of cell cycle were identified in the top 20 GO BP categories predicted as miRNA targets in our study. miR-1180-3p—the fourth marker validated in TCGA dataset—has not been observed to be associated with HER2 status to date.

Interactions between miRNAs and ER in breast cancer are mutually interrelated. Estrogen receptor interferes with the miRNA processing pathway by targeting Drosha complex, Argonaut proteins, and Dicer [10]. On the other hand, multiple miRNAs regulate the activity and expression of ER in breast cancer, which may translate into responsiveness to hormonal treatment. In our cohort, we observed upregulation of miR-92a-3p, a member of the miR-17-92 cluster, in the ER(−)/PgR(+) group. Its associations with ERα are unclear, but it directly downregulates ERβ in breast cancer [31]. In the Norwegian Women and Cancer study, miR-92a-3p was upregulated in triple-negative carcinomas [32]. So far, few studies have investigated interactions between PgR and miRNAs. One of the mechanisms

of ER-dependent upregulation of PgR involves downregulation of miR-26a and miR-181a, which bind to *PGR* 3'UTR and repress its expression [33]. In line with this, Gilam et al. proposed that miR-181a, miR-23a, and miR-26b might be responsible for PgR downregulation in ER(+)/PgR(−) tumors [34], but this was not confirmed by our data. One preliminary study suggested miR-495 as a novel negative regulator of ER and PgR [35]. As the vast majority of ER(−)/PgR(+) breast cancer express low levels of PgR, it may suggest that in some cases miR-495-3p contributes to a lack of ER expression with retained low PgR expression.

ER(+)/PgR(−) tumors from our cohort demonstrated higher expression of miR-29c-3p, a member of an miRNA cluster recently connected with ER(+) luminal tumors, consisting also of miR-149, miR-375, and miR-26b [11]. Similarly, the levels of miR-30 family members positively correlate with ER and a lack of EGFR [29]. High expression of miR-30a is associated with a favorable response to tamoxifen and a longer progression-free survival [29]. Another identified miRNA—miR-141-3p—belongs to another cluster characteristic for ER(+) tumors together with miR-451 and miR-486 [36]. Interestingly, miR-141-3p has reciprocal interactions with PgR. Progesterone downregulates miR-141-3p leading to derepression of signal transducer and activator of transcription 5A (Stat5a), and subsequently to expansion of stem-like breast cancer cells [37]. On the other hand, depletion of miR-141-3p increases PgR levels, even in breast cancer cell lines where its expression is ER-dependent [37]. This suggests that miR-141-3p downregulation may be a crucial event in the maintenance of PgR expression in ER(-) tumors.

In 2009, Lowery et al. identified miRNA signatures predicting expression of ER, PgR, and HER2 in breast cancer [38]. They demonstrated an association between miR-520g, miR-377, miR-527-518a, and miR-520f-520c and PgR, whereas miR-342, miR-299, miR-217, miR-190, miR-135b, and miR-218 predicted ER expression. Our study investigated miR-342, miR-299, miR-135, miR-218, but we did not observe any significant differences in their levels between groups. Recent research indicates that some miRNAs may directly target and silence ER expression, e.g., miR-18a-5p and miR-222 [39], and thus may participate in the development of ER(−)/PgR(+) breast cancer. A recent study by Gorbatenko et al. demonstrated that p95HER2 induces miR-221/222 and miR-503, leading to decreased *ESR1* expression and enhanced invasion and migration [39]. Likewise, miR-18a-5p is upregulated in ER(−) tumors and decreases expression of ER by binding to its mRNA [40]. In the current study, we observed a trend of higher expression of both these miRNAs in the ER(−)/PgR(+) group, but these findings lacked statistical significance (data not shown). Other miRNAs identified as potentially upregulated in ER(−)/PgR(+) (miR-514b-5p and miR-424) and ER(+)/PgR(−) (miR-423-5p) have not been previously reported to show differential expression with regard to steroid hormones receptor profiles. The main interactions between the discussed miRNAs, ER, PgR, and HER2 are summarized in Figure 4.

Limitations:

This was a retrospective study enrolling a small, clinically heterogenous cohort determined by frequency of ER(−)/PgR(+) cancers in the population (~1% of all breast cancers). Moreover, our observations need further validation, as we were able to externally validate expression only of miRNAs overlapping between our study and TCGA data.

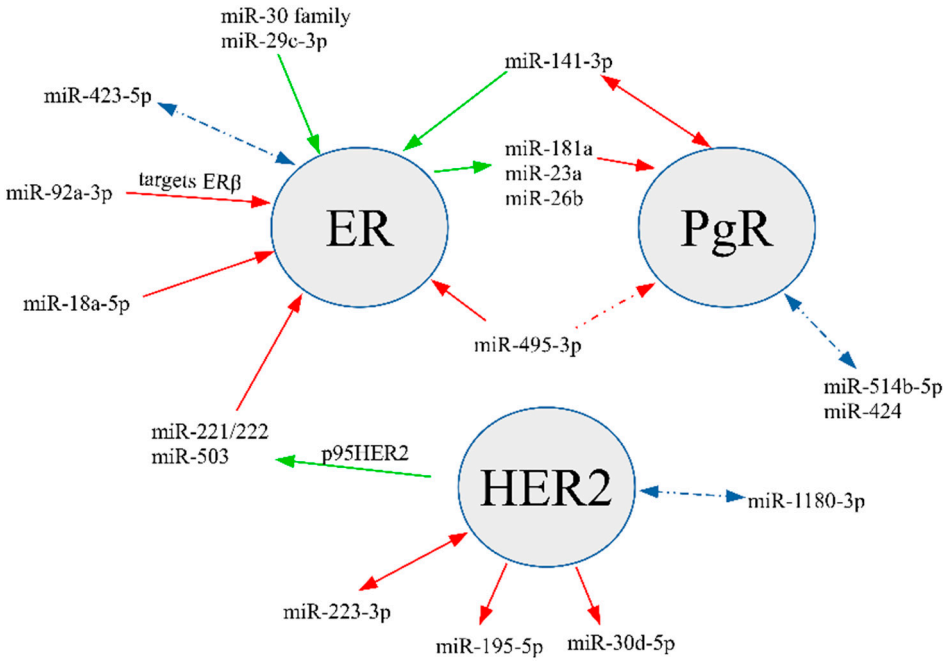

Figure 4. Key interactions between selected miRNAs, ER, PgR, and HER2. Red lines illustrate a known downregulating effect or an inverse association between miRNA and receptor; green lines depict an upregulating effect or a positive association between miRNA and receptor; blue lines show a potential relationship.

6. Conclusions

ER(−)/PgR(+) tumors show a profile resembling triple- and double-negative tumors, which may indicate that their biology is similar to basal-like carcinomas. On the contrary, ER(+)/PgR(−) tumors show a higher expression of miRNAs typical for double-positive luminal carcinomas. The main differences in miRNA expression amongst single hormone receptor-positive tumors were, however, related to their HER2 status. Further multicenter studies are necessary to comprehensively analyze miRNA signatures characteristic for ER(−)/PgR(+) and ER(+)/PgR(−) tumors.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4418/10/9/617/s1, **Supplementary Table S1.** List of patients (barcode IDs) analyzed for miRNA and mRNA expression in TCGA dataset along with ER/PgR/HER2 characterization of primary tumor; **Supplementary Table S2.** List of miRNA analyzed in NanoString and TCGA datasets (n = 185), including: HER2- (n = 33) and ER/PgR-associated (n = 8) miRNA in NanoString dataset; HER2- (n = 23) and ER/PgR-associated (n = 5) miRNA available for validation in TCGA dataset; miRNA names according to miRBase database v21 (NanoString) and v16 (TCGA); **Supplementary Table S3.** List of gene targets of HER2- (n = 33) and ER/PgR-associated (n = 8) miRNA in mammary gland tissue generated using miRNET 2.0 database; **Supplementary Table S4.** List of GO BP enriched in genes targeted by HER2-associated miRNAs in single hormone receptor positive breast tumors generated using Functional Annotation Tool by DAVID Bioinformatics Resources 6.81; **Supplementary Table S5.** List of GO BP enriched in genes targeted by ER/PgR-associated miRNAs in single hormone receptor positive breast tumors generated using Functional Annotation Tool by DAVID Bioinformatics Resources 6.81; **Supplementary Figure S1.** The chart presenting the number of miRNAs included in the study; **Supplementary Figure S2.** Distribution of clinicopathological characteristics of patients with single hormone receptor positive breast tumors in NanoString (n = 32) and TCGA cohorts (n = 67); differences estimated with t-test (age) or Fisher's Exact test (HER2 status, T, N, M); **Supplementary Figure S3.** Expression of HER2-associated miRNAs according to the ER/PgR/HER2 status (E—ER; P—PgR); expression depicted as number of counts of each probe normalized to all miRNA genes; differences estimated with Two-Way ANOVA (p-value) with Benjamini–Hochberg correction (FDR); bars correspond to IQR, whiskers cover 1.5 IQR from the median; **Supplementary Figure S4.** Volcano plot of HER2-associated miRNAs in single hormone receptor-positive breast tumors in NanoString cohort; for each miRNA -log10(p-value) plotted against log2FC; miRNAs upregulated in HER2(+) tumors marked in red, miRNAs downregulated in HER2(-) tumors marked in blue; p-value cut-off (-log10(p-value) = 1.45; matching FDR = 0.19) represented by a grey horizontal line; up/downregulation cut-offs (log2FC > 0.3 and log2FC < −0.3, respectively) represented by grey vertical lines; **Supplementary Figure S5.** Expression of ER/PgR-associated miRNAs according to the ER/PgR/HER2 status (E—ER; P—PgR); expression depicted as number of counts of each probe normalized

to all miRNA genes; differences estimated with Two-Way ANOVA (*p*-value) with Benjamini–Hochberg correction (FDR); bars correspond to IQR, whiskers cover 1.5 IQR from the median; **Supplementary Figure S6.** Volcano plot of ER/PgR-associated miRNAs in single hormone receptor positive breast tumors in NanoString cohort; for each miRNA -log10(*p*-value) plotted against log2FC; miRNAs upregulated in ER(+)/PgR(−) tumors marked in red, miRNAs downregulated in ER(+)/PgR(−) tumors marked in blue; *p*-value cut-off represented by grey horizontal line; up/downregulation cut-offs (log2FC > 0.3 and log2FC < −0.3, respectively) represented by grey vertical lines; **Supplementary Figure S7.** Reciprocal expression of ER/PgR-associated miRNA and their mRNA targets in TCGA dataset; for each of top20 GO BP terms enriched in NanoString cohort, mRNA targets of ER/PgR-associated miRNAs (5/8 available in TCGA dataset) were matched with genes associated with given term and the expression of each miRNA was correlated with expression of its mRNA targets; for each GO BP term, the overlap between mRNA correlating with miRNA of interest (cor > 0.3 or cor < −0.3) was illustrated with a Venn diagram; GO BP terms with at least 5 mRNA targets per each miRNA are presented (12/20, A-L); **Supplementary Figure S8.** mRNA differentiating ER/PgR phenotypes of breast tumors in TCGA dataset; transcriptome profiles of four ER/PgR phenotypes were compared and a list of differentiating genes (log2FC > 1 or log2FC < −1; FDR < 0.05, *t*-test) was generated for each comparison; the overlap between mRNA differentiating each pair of ER/PgR phenotypes was illustrated with a Venn diagram; **Supplementary Figure S9.** Correlation between expression of ER/PgR-associated miRNA and targeted mRNA that were uniquely differential for ER(+)/PgR(−) from ER(−)/PgR(+) breast tumors in TCGA dataset; miRNA-mRNA correlation was estimated for four available miRNA-mRNA pairs using Pearson's method; ER(+)/PR(−) tumors are marked in red; ER(−)/PR(+) tumors are marked in blue.

Author Contributions: Conceptualization, M.K., E.S.-K.; Data curation, M.P., A.S., M.N. and M.B. (Michał Bieńkowski).; Formal analysis, M.P., M.N., and M.B (Michał Bieńkowski); Funding acquisition, M.K. and E.S.-K.; Investigation, M.K., M.P., M.B. (Michał Bieńkowski), R.P. and E.S.-K.; Methodology, M.P., A.S., M.N., M.B. (Michał Bieńkowski), R.P. and A.K.; Project administration, E.S.-K.; Resources, M.K., A.S., R.P., A.Ł., M.B. (Marcin Braun), B.S.R., J.P., M.L., K.P., E.I.Ś., A.K., A.J.Ż. and W.B.; Software, M.P., M.B. (Michał Bieńkowski) and M.N.; Supervision, A.K., A.J.Ż., W.B. and E.S.-K.; Visualization, M.P., M.B. (Michał Bieńkowski); Writing—original draft, M.K., M.P. and E.S.-K.; Writing—review & editing, M.K., M.P., A.Ł., M.B. (Marcin Braun), B.S.R., J.P., M.L., K.P., E.I.Ś., A.J.Ż, W.B. and E.S.-K. All authors have read and agreed to the published version of the manuscript.

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CONTROVERSIES IN PATHOLOGY

High expression of progesterone receptor may be an adverse prognostic factor in oestrogen receptor-negative/progesterone receptor-positive breast cancer: results of comprehensive re-evaluation of multi-institutional case series

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Summary

Oestrogen receptor (ER)-negative (−) progesterone receptor (PgR)-positive (+) is the least common combination of steroid receptor expression observed in breast cancer. There are many controversies regarding the actual existence of ER−/PgR+ phenotype. In the current study, we aimed to perform comprehensive immunohistochemical re-evaluation of ER−/PgR+ breast cancers from multiple institutions. A total of 135 cases of ER−/PgR+ breast cancer were collected from 11 institutions from the period 2006−2020 and subsequently stained with three clinically validated anti-ER antibody clones: SP1 (Roche), 1D5 (Dako), and EP1 (Dako), and two anti-PgR antibody clones: 636 (Dako), and 1E2 (Roche). Clinicopathological characteristics of confirmed and re-categorised cases were analysed. Seventy-six cases retained the original ER−/PgR+ phenotype, including 21 HER2+ and 55 HER2− tumours. Forty-seven cases were ER+ with at least one anti-ER antibody, and 12 cases were re-categorised as double-negatives across all anti-ER and anti-PgR antibodies. No significant differences in survival were observed between groups in the HER2+ category. In the HER2− cohort, confirmed ER−/PgR+, ER+ tumours with discrepant ER staining, and triple negatives had inferior overall survival compared to concordant ER+ cases. Progesterone receptor expression in >20% of cells was identified as an adverse prognostic factor in ER−/PgR+/HER2− breast cancer in a multivariable model adjusted by stage (HR 5.0, 95% CI 1.3−19.2, p=0.019). We performed one of the largest validation studies so far on ER−/PgR+ breast cancer and confirmed the existence of this subgroup. Moreover, we identified high PgR expression as an adverse prognostic factor.

Key words: Oestrogen receptor; progesterone receptor; breast cancer; immunohistochemistry; prognosis.

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INTRODUCTION

The expression of progesterone receptor (PgR) in breast cancer is induced by oestrogen receptor α (ER). Therefore, the most common phenotype recognised in clinical practice is ER+/PgR+ (i.e., double-positive) breast cancer, whereas ER−/PgR+ phenotype is very infrequent. Multiple authors have neglected its existence or suggested an artifactual origin.[1–4] The frequency of ER−/PgR+ tumour diagnosis has decreased in recent years, probably due to improved immunohistochemistry (IHC) techniques.[5] The recommendations of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) advocate for repeated evaluation of ER expression from alternative blocks to reduce the risk of false negative results.[6] Nevertheless, even in restrictively controlled laboratories, ER−/PgR+ breast cancers are observed and pose a serious challenge for oncologists

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since no specific guidelines address the treatment of ER−/PgR+ breast cancer. Recently, with RNA sequencing analysis, Beltjens *et al.* demonstrated that ER−/PgR+ breast cancer is molecularly similar to triple negative breast cancer.[7] Up-regulation of the suppressor of zest 12 (SUZ12) may be a driver of the aggressive phenotype in these tumours.[7] A few small studies showed they were sensitive to both endocrine treatment and chemotherapy.[8,9] Moreover, PgR+ status in ER− tumours influences the current American Joint Committee on Cancer (AJCC) staging of breast cancer.[10]

To date, only a few studies have aimed to comprehensively re-evaluate ER−/PgR+ breast cancers.[3,4,11–13] Unfortunately, their results are contradictory and, at least partially, dependent on the anti-ER antibody used. Only one study analysed single cases of ER−/PgR+ breast cancer with three ER assays available for commonly used autostainer vendors (1D5 mixed with ER-2-123 in Dako, 6F11 in Leica, SP1 in Ventana) and demonstrated substantial differences, with no concordant case across all three analysed samples.[14] In the current retrospective multicentre study, we aim to reappraise diagnoses of ER−/PgR+ breast cancers collected from Polish and Hungarian centres, to identify the causes of misdiagnoses, and to analyse the clinical behaviour of any confirmed ER−/PgR+ breast cancers.

MATERIALS AND METHODS

Study group

The core group of patients was identified in the medical records of the University Clinical Center in Gdańsk (UCCG) using the MedStream Designer tool in the period from 2006 to 2020. In the other centres, cases were identified in the local databases and sent for central evaluation. Basic clinicopathological data (age, TNM stage, grade, HER2 status, Ki67 expression) and information concerning patients' clinical course (type of treatment, presence of relapse, and/or death) were collected, if available. Finally, 151 cases of ER−/PgR+ tumour were collected from nine Polish and two Hungarian centres. Study exclusion criteria comprised cases lacking tissue samples unexposed to systemic therapy (n=14), cases with hormone receptor (HR) status established only in the nodal or distant metastases (n=0), and lacking invasive component (n=2).

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from 135 preselected cases consisted of 86 core needle or vacuum assisted breast biopsies and 76 post-operative samples. In 27 cases both pre- and post-operative material was available for comparison. The study was approved by the Bioethical Committee of the coordinating centre, the Medical University of Gdansk, Poland (approval no: NKBBN/119/2018). All research was performed in accordance with the appropriate regulations.

Immunohistochemistry

All cases enrolled in the study (including those re-categorised after evaluation of primary IHC) were subsequently stained with three antibody clones against ER [1D5, Dako, Denmark; EP1 (routinely used in UCCG laboratory), Dako; SP1, Roche, Switzerland], and anti-PgR [clone 636 (routinely used in UCCG laboratory), Dako] according to the manufacturer's guidelines. To save available tissue material only cases with consistent ER− status across three antibody clones and PgR− or equivocal status obtained with anti-PgR 636 clone were additionally stained with anti-PgR 1E2 clone (Roche). The antibodies not routinely used in our laboratory were validated with breast cancer cases with known ER and PgR IHC status before the study. Characteristics of all utilised antibodies are shown in Table 1. If available, more than one tissue block was used to obtain material for staining (average 2.02 blocks/case, range 1–6). Internal positive controls for ER and PgR expression were identified in all cases (non-neoplastic glandular elements adjacent to cancer). Nuclear staining in >1% of cells was considered positive.[6]

mRNA biomarker assessment

Additionally, in five selected cases that retained ER−/PgR+ status after all steps of re-evaluation, an ESR1/PGR/ERBB2/MKi67 mRNA biomarker assessment was performed to objectify the findings using Xpert Breast Cancer STRAT4 (Cepheid, USA). Briefly, this system is a multiplexed reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) for the quantitative assessment of four breast cancer biomarkers utilising the single use cartridges. FFPE tissue blocks containing cancer tissue were cut into four 20 µm slices and then a lysate was prepared using an FFPE Lysis Kit (Cepheid), added to the cartridge, and placed into the GX instrument.

Statistics

Categorical variables were compared by Fisher's exact test or Chi-square test with Yates correction. The normal distribution of the data was assessed using the Shapiro–Wilk test. Continuous variables were analysed utilising the Mann–Whitney U test or Kruskal–Wallis test when applicable. The agreement between clones of anti-ER antibody was assessed by Fleiss' kappa and Cohen's kappa coefficients.

Overall survival (OS) was defined as the time from the diagnosis to the date of death from any cause. Kaplan–Meier curves were plotted to calculate the survival rates of ER−/PgR+ and other phenotypes. Hazard ratios (HRs) were estimated for the different phenotype groups with reference to ER+/PgR+ using Cox regression analysis. A p value <0.05 was considered significant; in cases of multiple comparisons p values were adjusted at a false discovery rate (FDR)=0.05 using Benjamini–Hochberg correction.

Statistical analyses were performed with the use of Statistica 13 (RRID:SCR_014213, Tibco, CA, USA) licensed to Medical University of Gdańsk, and R statistical environment.[15] Boxplots and scatterplots were generated using the 'ggplot2' package.[16] Kaplan–Meier curves were plotted using the 'survminer' and 'ggsci' packages.[17,18]

RESULTS

Immunohistochemistry

The detailed characterisation of the study group is shown in Table 2 and the study flowchart is shown in Fig. 1. After primary evaluation, the total number of 135 breast cancer cases primarily designated as ER−/PgR+ were enrolled in the IHC study. Eventually, within this group, 76 cases (56.3%) retained the original phenotype, in 47 (34.8%) the status was

Table 1 Basic characteristics of anti-ER and anti-PgR antibodies used in the study

Antigen	Antibody	Manufacturer	Host	Clonality	Autostainer	Concentration
ER	1D5	Dako	Mouse	Monoclonal	Dako	Ready-to-use
ER	SP1	Roche	Rabbit	Monoclonal	Ventana	Ready-to-use
ER	EP1	Dako	Rabbit	Monoclonal	Dako	Ready-to-use
PgR	636	Dako	Mouse	Monoclonal	Dako	Ready-to-use
PgR	1E2	Roche	Rabbit	Monoclonal	Ventana	Ready-to-use

ER, oestrogen receptor, PgR, progesterone receptor.

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Table 2 The clinicopathological characteristic of the study group

Characteristic	Whole group	Confirmed ER−/PgR+	ER+/PgR+ and ER+/PgR−	ER−/PgR−	*p* value
No. cases	135	76	47	12	
Age, years, median (IQR)	56 (51–57)	60 (51–67.5)	49 (42–60)	62.5 (44–76)	0.278
Tumour size, mm, median (IQR)	25 (17–41)	30 (16–45)	24.5 (16.5–33.5)	25 (20–45)	0.095
Histology					
Ductal	127 (94%)	72 (95%)	43 (91%)	12 (100%)	0.847
Lobular	4 (3%)	2 (2.5%)	2 (4.5%)	0 (0%)	
Other	4 (3%)	2 (2.5%)	2 (4.5%)	0 (0%)	
T					
1	47 (35%)	25 (33%)	19 (40%)	3 (25%)	0.444 (T1–T2 vs T3–T4)
2	55 (41%)	29 (38%)	19 (40%)	7 (58%)	
3	18 (13%)	13 (17%)	5 (11%)	0 (0%)	
4	11 (8%)	6 (8%)	3 (6%)	2 (17%)	
Missing	4 (3%)	3 (4%)	1 (2%)	0 (0%)	
N					
0	73 (54%)	42 (55%)	26 (55%)	5 (42%)	0.644 (N0 vs N1–3)
1	38 (28%)	19 (25%)	14 (30%)	5 (42%)	
2	15 (11%)	9 (12%)	5 (11%)	1 (8%)	
3	6 (4%)	3 (4%)	2 (4%)	1 (8%)	
Missing	3 (2%)	3 (4%)	0 (0%)	0 (0%)	
M					
0	125 (93%)	68 (89%)	45 (96%)	12 (100%)	0.359
1	6 (4%)	5 (7%)	1 (2%)	0 (0%)	
Missing	3 (%)	3 (4%)	1 (2%)	0 (0%)	
Grade					
1	3 (2%)	1 (2%)	2 (4%)	0 (0%)	0.005
2	38 (28%)	14 (18%)	22 (47%)	2 (17%)	
3	94 (70%)	61 (80%)	23 (49%)	10 (83%)	
PgR%					
<20%	77 (57%)	49 (64%)	28 (60%)	NA	0.345
>20%	46 (34%)	27 (36%)	19 (40%)	NA	
HER2					
Negative	93 (69%)	55 (72%)	32 (68%)	6 (50%)	0.295
Positive	42 (31%)	21 (28%)	15 (32%)	6 (50%)	
BRCA1/2 status					
Negative/Unknown	120 (89%)	65 (86%)	44 (94%)	11 (92%)	0.362
Positive	15 (11%)	11 (14%)	3 (6%)	1 (8%)	
Preoperative chemotherapy					
No	81 (60%)	42 (55%)	34 (72%)	5 (42%)	0.068
Yes	54 (40%)	34 (45%)	13 (28%)	7 (58%)	
Preoperative endocrine therapy					
No	132 (98%)	74 (97%)	46 (98%)	12 (100%)	0.846
Yes	3 (2%)	2 (3%)	1 (2%)	0 (0%)	
Adjuvant chemotherapy					
No	56 (41.5%)	37 (49%)	14 (30%)	5 (42%)	0.358
Yes	55 (40.5%)	29 (38%)	19 (40%)	7 (58%)	
Missing	24 (18%)	10 (13%)	14 (30%)	0 (0%)	
Adjuvant endocrine therapy					
No	74 (55%)	44 (58%)	20 (42%)	10 (83%)	0.359
Yes	37 (27%)	22 (29%)	13 (28%)	2 (17%)	
Missing	24 (18%)	10 (13%)	14 (30%)	0 (0%)	
Type of material					
Biopsy	59 (44%)	42 (55%)	13 (28%)	4 (43%)	0.001
Biopsy/post-op	27 (20%)	12 (16%)	9 (19%)	6 (50%)	
Post-op	49 (36%)	22 (29%)	25 (53%)	2 (17%)	
Relapse/progression					
No	112 (83%)	65 (86%)	38 (81%)	9 (75%)	0.594
Yes	23 (17%)	11 (14%)	9 (19%)	3 (25%)	
Death					
No	112 (83%)	64 (84%)	38 (81%)	10 (83%)	0.889
Yes	23 (17%)	12 (16%)	9 (19%)	2 (17%)	

p value calculated with chi square test.
ER, oestrogen receptor; IQR, interquartile range; NA, not applicable; PgR, progesterone receptor.

changed to ER+, and 12 (8.9%) tumours were double-negatives. The examples of confirmed ER−/PgR+ breast cancer are shown in Fig. 2 and Fig. 3.

Discordant expression of ER in various tissue blocks occurred in three cases (2.2%), leading to the change of a phenotype to ER+/PgR+. This may suggest that tumour heterogeneity is rarely accountable for a single hormone receptor phenotype. It is further supported by the finding that in all confirmed ER−/PgR+ breast cancers with core needle biopsy and chemo-naïve resection specimen available for comparison, the results were concordant. Additionally, we compared the group with only one FFPE tissue block available and the group with >1 block analysed, and no statistically significant difference between these groups was noted in the frequency of confirmed ER−/PgR+ cases (63% vs 48.5%, p=0.138, chi-square).

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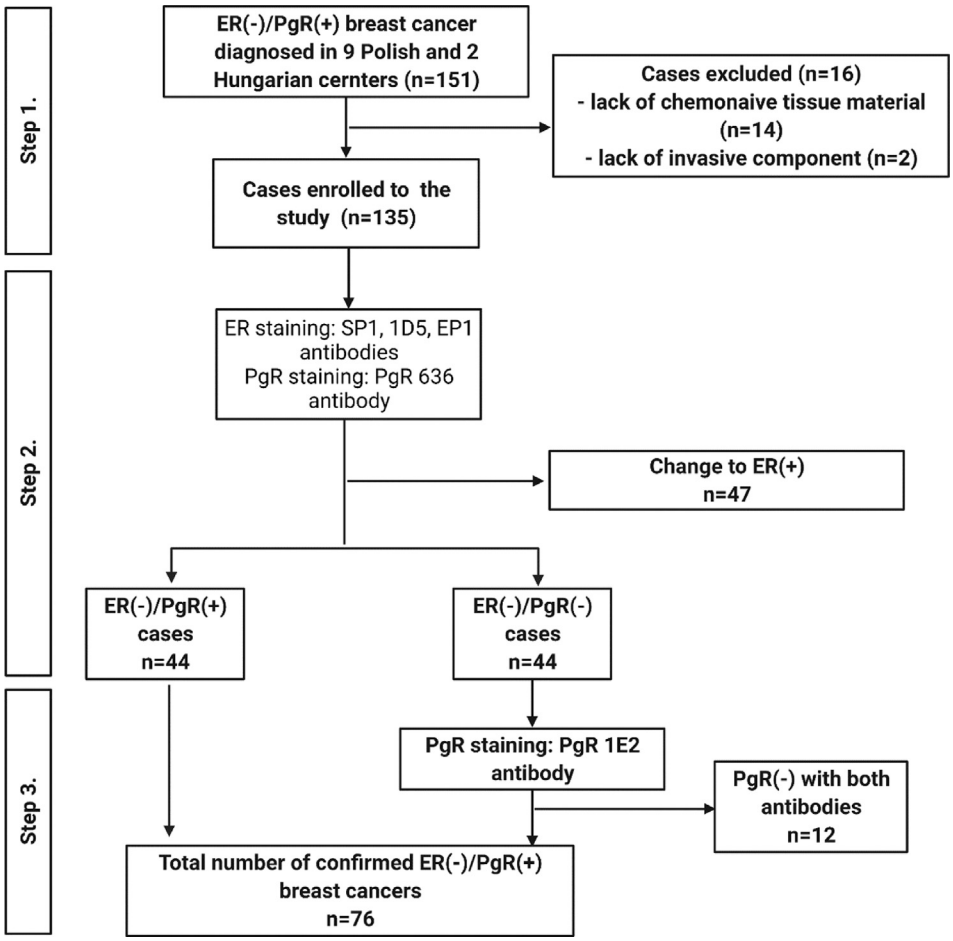

Fig. 1 Flowchart of the study.

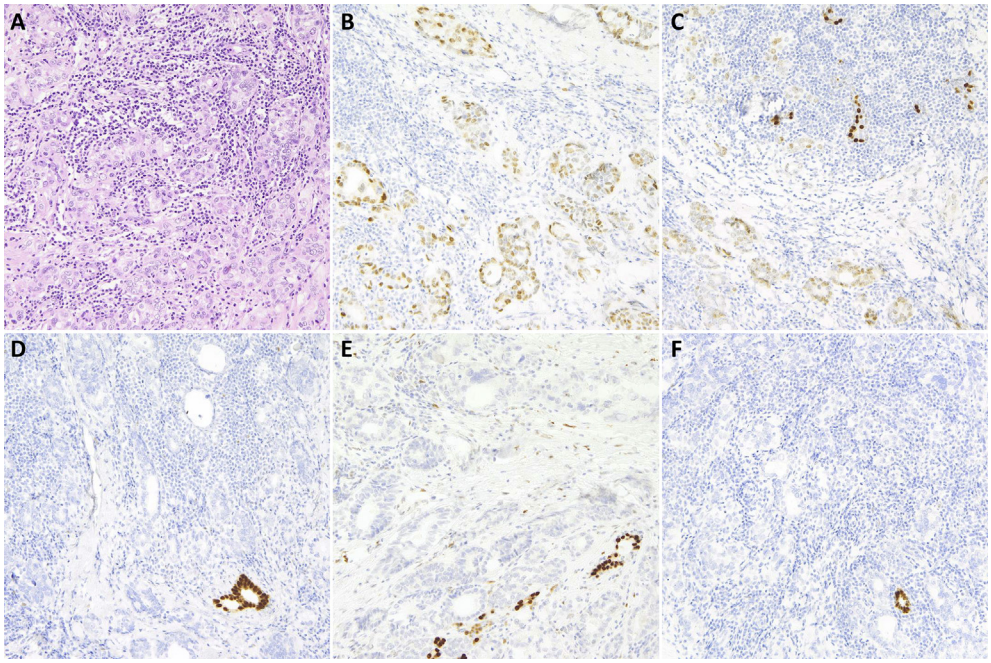

Fig. 2 Example of confirmed ER−/PgR+ breast cancer. (A) Neoplastic tubules scattered in the lymphocyte-rich stroma in haematoxylin and eosin staining; (B) positive PgR 636 staining with (C) positive control; negative ER stainings with positive internal controls, (D) EP1, (E) SP1, (F) 1D5. ER, oestrogen receptor; PgR, progesterone receptor.

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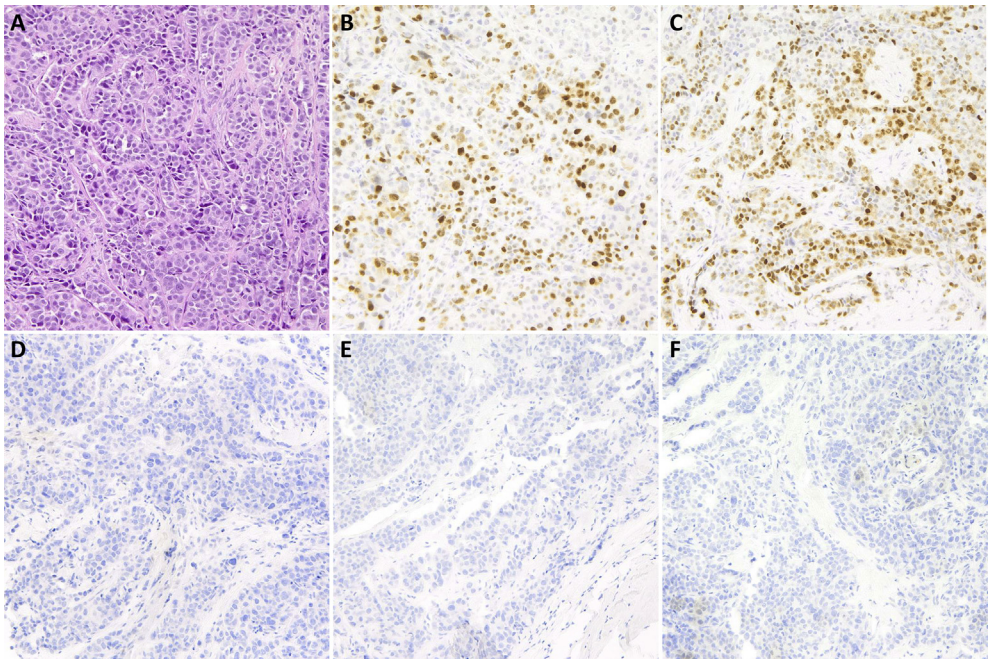

Fig. 3 Example of confirmed ER−/PgR+ breast cancer. (A) Core needle biopsy of high grade infiltrating duct carcinoma in haematoxylin and eosin staining; (B,C) positive PgR 636 staining; negative ER stainings with (D) EP1, (E) SP1, (F) 1D5 (F). Internal positive control for ER was detected in this specimen with all clones. ER, oestrogen receptor; PgR, progesterone receptor.

We observed non-negligible discrepancies in results of ER staining, but the general agreement in binary classification into negative or positive expression across three investigated clones was substantial (Fleiss' kappa 0.73). The worst concordance was observed between the SP1 and EP1 clones (Table 3). Similarly, a correlation between the percentage of positively staining nuclei was high between all three antibodies, but the correlation was the weakest for SP1 and EP1 (Fig. 4). Overall, discordant stainings were present in 21 (15.5%) of tumours. Positive concordance across three antibody clones was observed in 26 (19.3%), and negative in 88 (65.18%) cases.

We also observed a discrepancy in PgR staining. Of 42 PgR− cases by 636 clone, staining with 1E2 clone demonstrated positive nuclear reaction in 32 (76.2%). Eventually, 76 tumours showed concordant ER− phenotype with three anti-ER antibody clones and PgR expression in at least one anti-PgR clone. The examples of discrepant ER and PgR stainings are shown in Fig. 5.

Further proof of the existence of the ER−/PgR+ phenotype was obtained at the molecular level. Xpert Breast Cancer STRAT4 confirmed the ER−/PgR+ phenotype in four of five cases in which the analysis was performed (Fig. 6). A single case unconfirmed in mRNA assay (ER/PgR negative in the mRNA analysis) had 15% of PgR+ nuclei in IHC staining.

Table 3 The concordance between anti-ER antibodies in the binary classification of ER expression (negative versus positive)

	ER 1D5+	ER 1D5−	Cohen's kappa (95% CI)
ER SP1+	33	12	0.786 (0.673−0.899)
ER SP1−	0	90	
	ER EP1+	ER EP1−	
ER SP1+	26	19	0.613 (0.470−0.757)
ER SP1−	2	88	
	ER 1D5+	ER 1D5−	
ER EP1+	26	2	0.810 (0.691−0.929)
ER EP1−	7	100	

Clinicopathological features of confirmed ER−/PgR+ cases

The vast majority of confirmed ER−/PgR+ cases (n=61, 80.3%) were classified as grade 3, and almost all of them (n=72, 94.7%) exhibited morphology of infiltrating duct carcinoma of no special type (Table 2). HER2 overexpression or amplification was detected in 21 tumours (27.6%). The highest percentage of PgR+ cells was observed in ER+/PgR+/HER2− tumours, whereas ER−/PgR+/HER2− and discordant ER+/PgR+/HER2− breast cancers were characterised by significantly lower percentage of PgR+ cells (Fig. 7A). No significant differences in the percentage of PgR+ cells were noted in the HER2+ group but the number of cases in this subgroups was low (Fig. 7B).

Within the confirmed ER−/PgR+ subgroup, patients most frequently presented with T2 tumours (n=29, 38.2%). Nodal metastases were observed in 31 patients (40.8%), and five patients (6.6%) manifested *de novo* distant metastatic disease. Almost all tumours with available Ki67 results displayed a very high proliferation index (median 60%). Thirty-four (44.7%) patients received pre-operative chemotherapy, and 12 tumours (35.3%) achieved complete pathological response (pCR). The response rate was not statistically different from re-categorised (ER+ and/or PgR−) groups.

In the whole cohort, 37 patients were treated with adjuvant endocrine therapy (27.4%), including 22 patients (28.9%) with confirmed ER−/PgR+ diagnoses. Adjuvant chemotherapy was administered to 55 patients (40.7%) in the whole cohort, including 29 confirmed ER−/PgR+ cases (38.2%). Pre-operative and post-operative anti-HER2 treatment was administered to nine patients (6.7%) and 28 patients (20.74%), respectively. In the confirmed ER−/PgR+/HER2+ group, anti-

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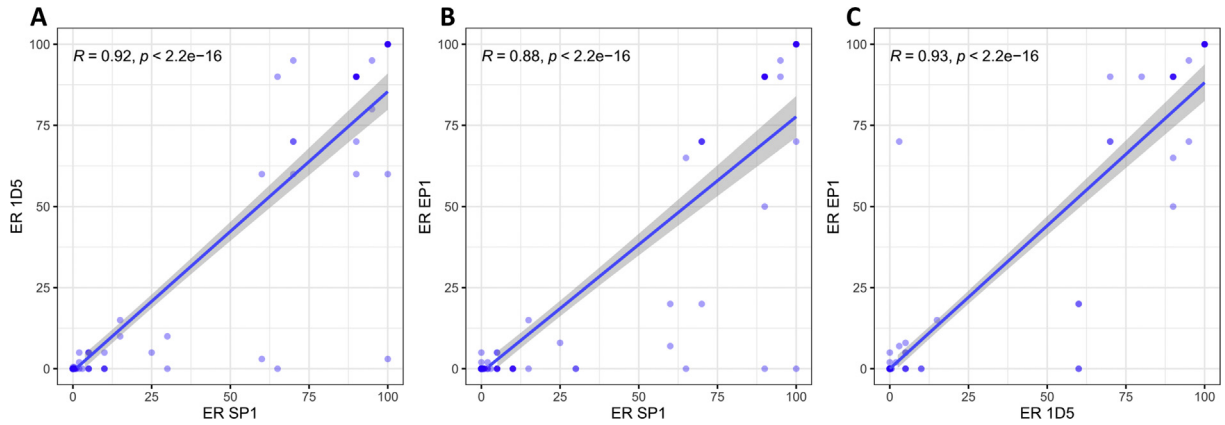

Fig. 4 Correlation between percentage of positively staining cells between three clones of anti-ER antibody. ER, oestrogen receptor; R, Spearman's rank correlation coefficient.

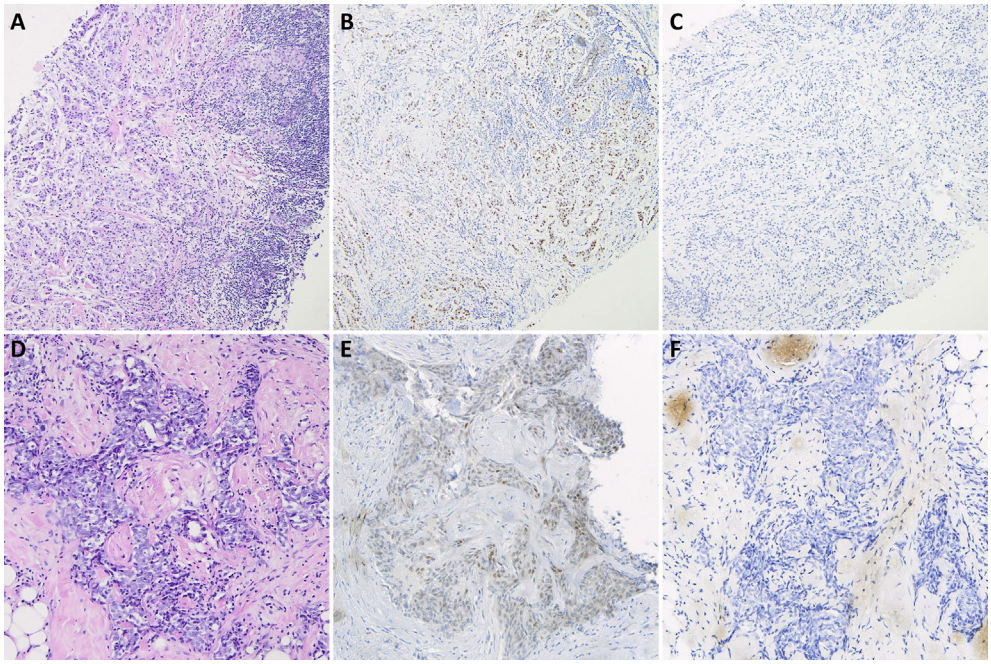

Fig. 5 Examples of discrepant ER and PgR stainings. (A–C) First case: (A) haematoxylin & eosin staining, (B) positive PgR 1E2 staining, and (C) negative PgR 636 staining. (D–F) Second case: (D) haematoxylin & eosin staining, (E) weakly positive ER SP1 staining, and (F) negative ER EP1 staining. ER, oestrogen receptor; PgR, progesterone receptor.

HER2 treatment was applied in the neoadjuvant setting to 5/21 patients (23.8%), and post-operatively in 15/21 patients (71.4%; information was missing for 3 patients, 14.3%).

Survival of confirmed ER−/PgR+ breast cancer compared to re-categorised cases

The OS was 81.3% in the whole group with a median follow-up time of 44 months [interquartile range (IQR) 27–59 months). To assess clinical consequences of ER−/PgR+ breast cancer reclassification we performed survival analyses comparing confirmed and recategorised cases.

In the group of HER2− breast cancer, the best outcomes were observed in patients with ER+ status confirmed by three anti-ER antibodies (Fig. 8A). Confirmed ER−/PgR+ cases, triple-negative cancers, and ER+ tumours with discordant staining results (lacking agreement across all anti-ER clones) had a similar clinical course with inferior outcomes (Table 4).

In the group of HER2+ tumours we did not observe any significant differences in survival, but a smaller number of cases might have influenced the results, and precluded calculation of HRs. Nevertheless, we observed a trend toward better prognosis in HER2-overexpressing/amplified ER−/PgR+ breast cancer when compared to ER−/PgR+/HER2− cases with borderline statistical significance (log-rank p=0.067; HR=0.20, 95% confidence interval 0.03–1.605) (Fig. 8B).

Regarding treatment modalities, in the group of confirmed ER+/PgR− cases, we did not find any statistically significant effects on OS taking into consideration pre-operative chemotherapy (HR=0.98, 95% CI 0.31–3.11, p=0.97), post-operative chemotherapy (HR=0.90, 95% CI 0.26–3.13, p=0.874), and post-operative endocrine therapy (HR=0.44, 95% CI 0.09–2.14, p=0.312). No death was noted amongst patients treated with anti-HER2 drugs in adjuvant setting (HR impossible to calculate).

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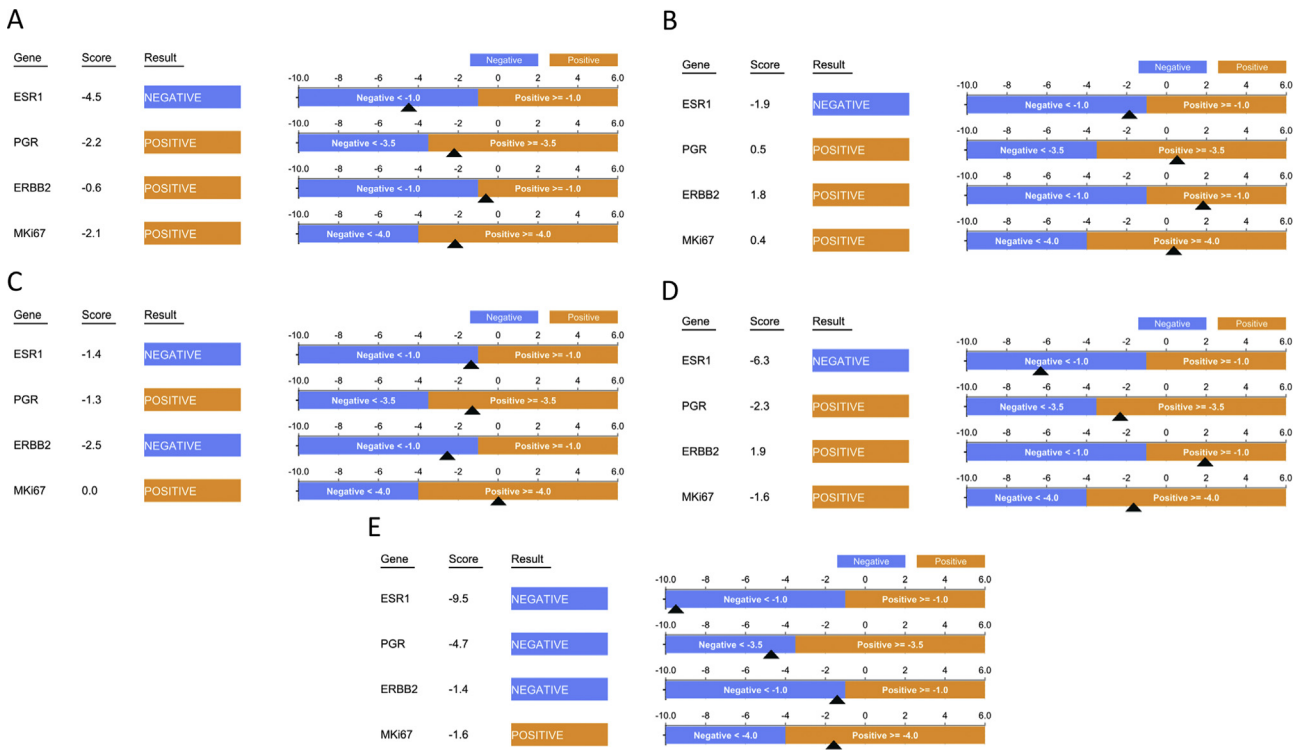

Fig. 6 (A–E) The results of Xpert Breast Cancer STRAT4 assay in five ER–/PgR+ breast cancer cases confirmed by immunohistochemistry. (E) The case negative in STRAT4 assay immunohistochemistry revealed positive PgR staining in 15% of nuclei. ERBB2, Erb-B2 receptor tyrosine kinase 2; ESR1, oestrogen receptor 1; MKi67, marker of proliferation Ki-67; PGR, progesterone receptor.

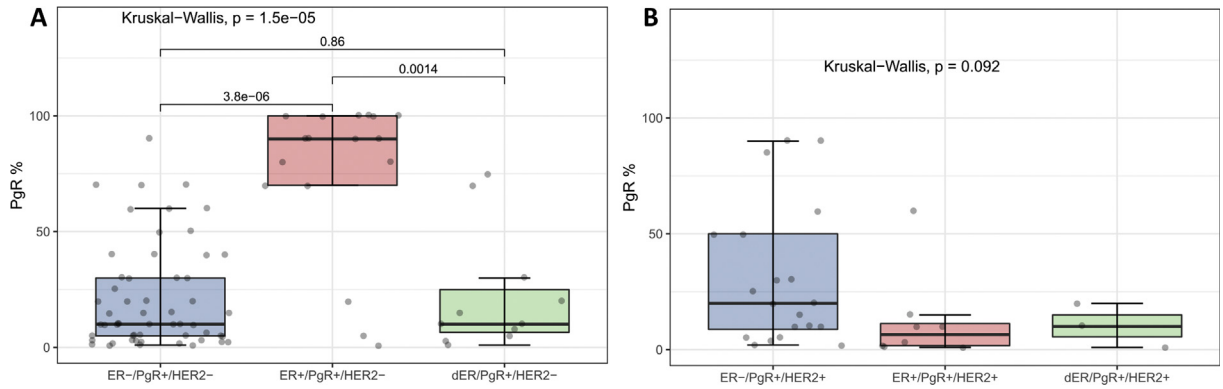

Fig. 7 Percentage of PgR+ cells according to the phenotype of (A) HER2– and (B) HER2+ breast cancer. The boxplot displays the median values of the percentage of PgR+ cells, which is indicated by the centreline with a dot. The edges of the boxes represent the 25th percentile and 75th percentile. The horizontal lines outside the boxes show the maximum and minimum values of the data that are within 1.5 times the interquartile range over the 75th and 25th percentile, respectively. Dots depict individual measures. D, discordant staining; ER, oestrogen receptor; PgR, progesterone receptor.

In the next step, we assessed the prognostic significance of the PgR expression level on survival among ER–/PgR+/HER2– tumours. The receiver operating curve was plotted to find the cut-off value discriminating patients in terms of fatal outcomes (Fig. 9). Surprisingly, breast cancers expressing PgR in >20% of cells showed inferior OS (Fig. 10A). This effect was retained utilising either local or central assessment of the PgR expression. Moreover, there was no difference in OS between PgR 636+ and PgR 1E2+ only tumours. As the stage is the crucial prognostic factor in breast cancer, we evaluated the prognostic impact of the percentage of PgR-expressing cancer cells in the multivariable Cox regression model adjusted by stage (Table 5). The status of PgR retained its statistical significance in multivariate analysis. The opposite trend (without statistical significance) was observed in ER+/PgR+ group, in which a higher percentage of PgR+ cells was associated with longer OS (Fig. 10B).

DISCUSSION

ER–/PgR+ breast cancer is the most controversial breast cancer subtype defined by hormone receptor status. This is the first approach to assess these cancers in Poland. In Hungary, a previous study confirmed the ER–/PgR+ phenotype in only one case from a cohort of 182 cases.[12]

The frequency of ER–/PgR+ cancer diagnosis has decreased in recent years and stabilised in most countries at the level of approximately 1.0–1.5%. According to the data

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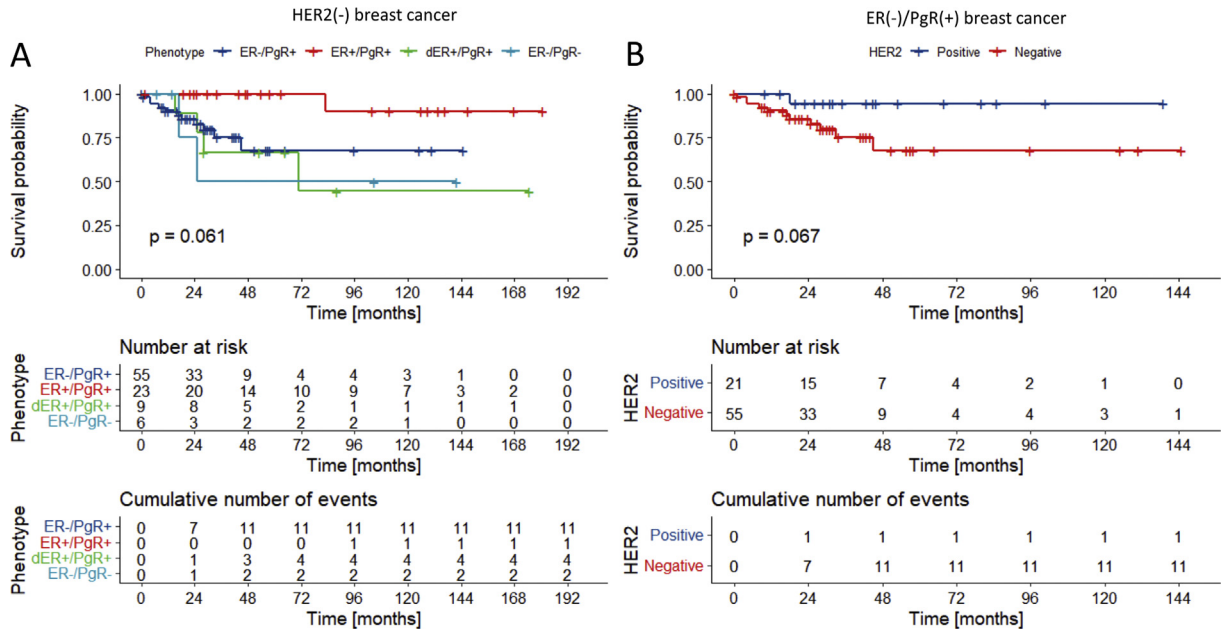

Fig. 8 (A) Overall survival of HER2− breast cancers divided by ER and PgR status, including discordant ER+ group; (B) overall survival of ER−/PgR+ breast cancer stratified by HER2 status. Demonstrated *p* values were calculated with a log-rank test. D, discordant staining; ER, oestrogen receptor; PgR, progesterone receptor.

Table 4 The results of univariate Cox proportional hazard analysis in HER2− breast cancer in reference to ER+/PgR+ group

Phenotype	HR	95% CI	*p* (Cox)	Adjusted *p* (Cox)	*p* (log-rank)	Adjusted *p* (log-rank)
ER+ with concordant results	1.000	N/A	N/A	N/A	N/A	N/A
ER+ with discordant results	12.343	1.357–112.19	0.025	0.075	0.006	0.018
ER−/PgR+	8.314	1.035–66.77	0.046	0.069	0.015	0.022
ER−/PgR−	11.186	1.012–123.680	0.048	0.048	0.024	0.024

Adjusted *p* values were calculated with Benjamini–Hochberg correction.
CI, confidence interval; ER, oestrogen receptor; HR, hazard ratio; PgR, progesterone receptor.

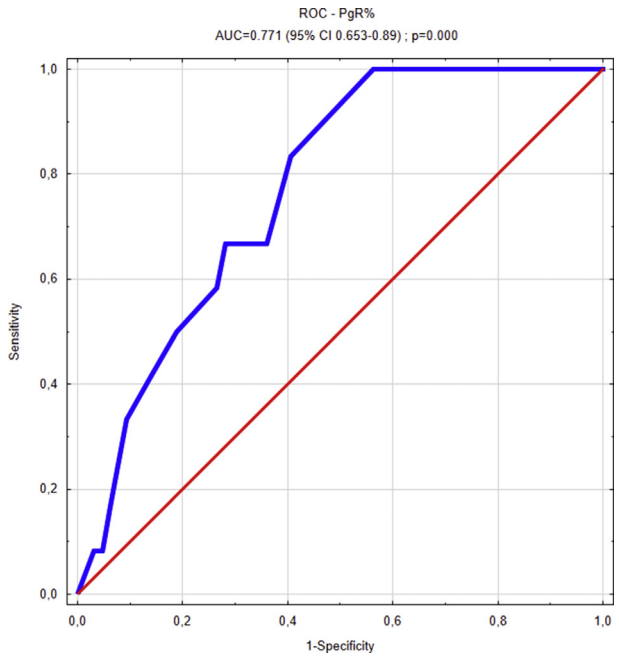

Fig. 9 Receiver operating curve for the percentage of PgR+ cancer cells plotted against death. AUC, area under curve; CI, confidence interval; ROC, receiver operating curve.

from Surveillance, Epidemiology, and End Results (SEER) in the USA, the drop in ER−/PgR+ phenotype has been noted since 1989.[19] These changes are believed to be associated with the switch from the ligand-binding assay to IHC and subsequent improvements in IHC detection methods. Moreover, the threshold for ER positivity was reduced from 10% to 1%. Some authors postulate that ER−/PgR+ tumours should not be diagnosed if any nuclear staining for ER in cancer cells is present.[2,4] Hereby, we utilised 1% of reactive nuclei as a threshold, which is recommended by the current guidelines, and validated in clinical practice.[6]

We identified several possible causes of ER−/PgR+ misdiagnosis. One of the avoidable mistakes is a typo or mislabelling of the phenotype in the pathology report, e.g., designation of ER+/PgR− tumour as ER−/PgR+. Another possibility is a misinterpretation of cytoplasmic PgR expression as positive, counting entrapped non-neoplastic glandular PgR+ cells as cancer cells, and PgR expression in <1% of cells. In several cases, we observed weak and focal expression of PgR in stromal cells or immune cells. Finally, weak nuclear expression of ER in a low number of cells (1–10%) might have gone unnoticed in some cases or have been confused with non-neoplastic glands in well-differentiated tumours.

In two recent studies, ER−/PgR+ cancers showed worse prognosis than double-positive and ER+/PgR− tumours,

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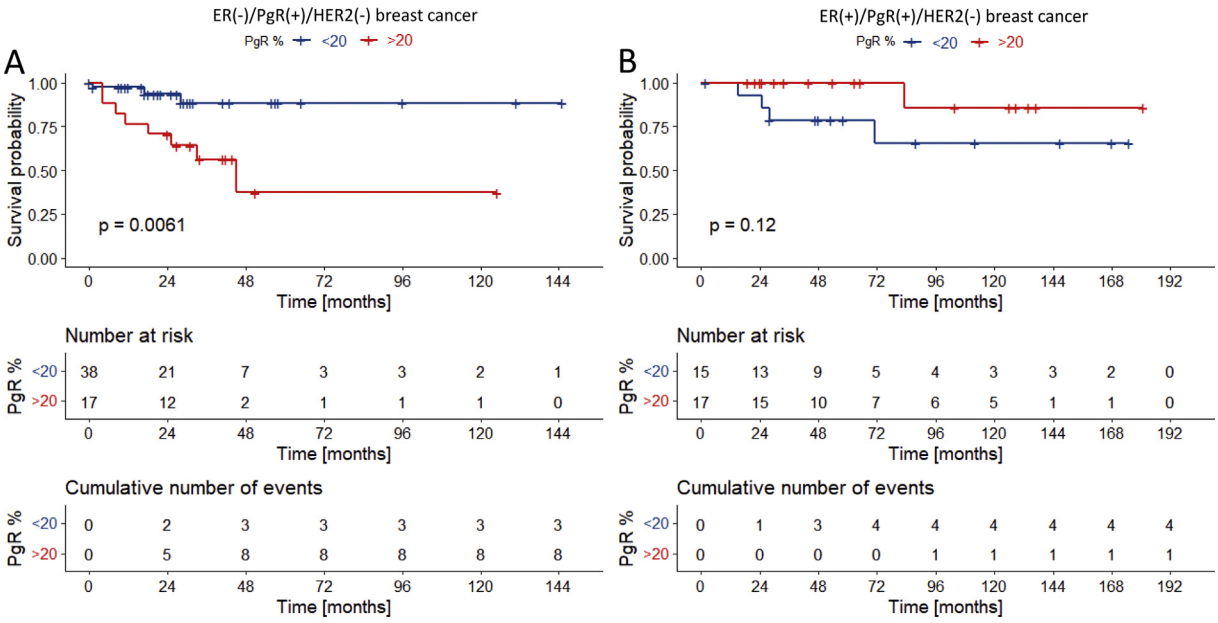

Fig. 10 (A) Overall survival of ER−/PgR+/HER2− breast cancer stratified by the percentage of PgR+ cells; (B) overall survival of ER+/HER2− breast cancer stratified by the percentage of PgR+ cells. Demonstrated *p* values were calculated with a log-rank test. ER, oestrogen receptor; PgR, progesterone receptor.

Table 5 Multivariable Cox regression model predicting survival in ER−/PgR+/HER2− breast cancer including stage and the percentage of PgR+ cells

Feature	HR	95% CI	*p*
Stage (3–4 vs 1–2)	3.8	1.1–12.9	0.033
PgR (>20% + cells vs <20% + cells)	5.00	1.3–19.2	0.019

CI, confidence interval; ER, oestrogen receptor; HR, hazard ratio; PgR, progesterone receptor.

which emphasises their distinctive aggressive biology.[20,21] Our results are consistent with these findings. Thus, we conclude that the tumours with positive ER staining in <1% of cells and PgR+ in >1% of cells should not be considered double-positive cases, because of their distinctively poor outcomes. Moreover, the majority of these tumours present basal gene expression profiles in the PAM50 classifier, which coincides with their aggressive biology.[22] One of the most counterintuitive findings in our study was the association between higher expression of PgR and worse OS in ER−/PgR+/HER2− breast cancers. We hypothesise that in the absence of ER expression, PgR may drive the expression of a distinct subset of genes responsible for more aggressive biology. Alternatively, these cancers may display an imbalance in PgR isoforms leading to overexpression of PgR-A isoform, which characterises breast cancers with loss of endocrine sensitivity and poorer outcomes.[23,24] On the other hand, the trend towards a better prognosis in HER2+ cases is consistent with the recent study based on the SEER database.[25] This effect is most likely associated with benefits from trastuzumab treatment.

The higher sensitivity of the SP1 antibody compared to the 1D5 antibody is consistent with previous reports.[26,27] In the study comparing SP1, 6F11, and 1D5 antibodies, nine 1D5-negative cases demonstrated weak positivity for SP1 and/or 6F11.[27] Of note, these cancers were characterised by high grade, high Ki67 index, HER2 overexpression, and PgR negativity or low positivity, suggesting endocrine resistance.[27] Another study demonstrated that EP1 antibody has a high concordance with Dako ER/PR pharmDx kit and SP1 antibody, but the use of EP1 improved interpretation of ER IHC results.[28,29]

The very high percentage of PgR 636−, but 1E2+ cases is one more striking finding in our study. Troxell *et al.* reported 1E2 antibody generating more positive results in cases generally negative for PgR with other antibody clones.[30] As emphasised by Kornaga *et al.*, the Roche/Ventana assay recognises both isoforms of PgR, which may explain this discrepancy.[31] Moreover, the same study suggests that Roche/Ventana assay with 1E2 clone may have superior prognostic value compared to other vendors.[31] Nevertheless, we accept the possibility that some cases may represent false-positive staining.

Our findings suggest that a diagnosis of ER−/PgR+ breast cancer is frequently associated with high grade, low PgR positivity, and high Ki67 index. These findings are supported by the recent study of ER−/PgR+ tumours from Japan,[13] which concluded with a recommendation for retesting in particular low grade cases, and those with a high proportion of PgR positive cells. Special caution should be applied to biopsy specimens, which may represent a relatively small cancer area dominated by ER−/PgR+ cells; however, in our cohort full concordance was present between biopsy and post-operative material amongst confirmed ER−/PgR+ cases. Nevertheless, this problem is very difficult to overcome, due to the more and more frequent use of pre-operative systemic therapy, which may lead to complete tumour regression and lack of tissue material for comparison with a biopsy sample.

Our study has several limitations. Most importantly, the cohort was clinically heterogeneous and relatively small, which is dependent on the low frequency of ER−/PgR+ phenotype. Thus, the conclusions from survival analyses should be interpreted with caution. In multiple cases, only biopsy material was available for analysis. Moreover, to save valuable tissue material (especially from core needle biopsies) only PgR 636− tumours were stained with the 1E2 clone.

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CONCLUSIONS

Pathologists should be cautious in every case of ER−/PgR+ tumour, especially when demonstrating low-grade morphology, low Ki67 index, or lack of internal ER control. Preferentially, re-evaluation should be performed with an alternative FFPE block, and, if possible, with the use of another antibody clone. In doubtful cases, mRNA assays, e.g., STRAT4, may be used to confirm the diagnosis. Further multicentre studies are necessary to establish the molecular landscape of these rare cancers, hopefully leading to the identification of new targets for personalised therapy.

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