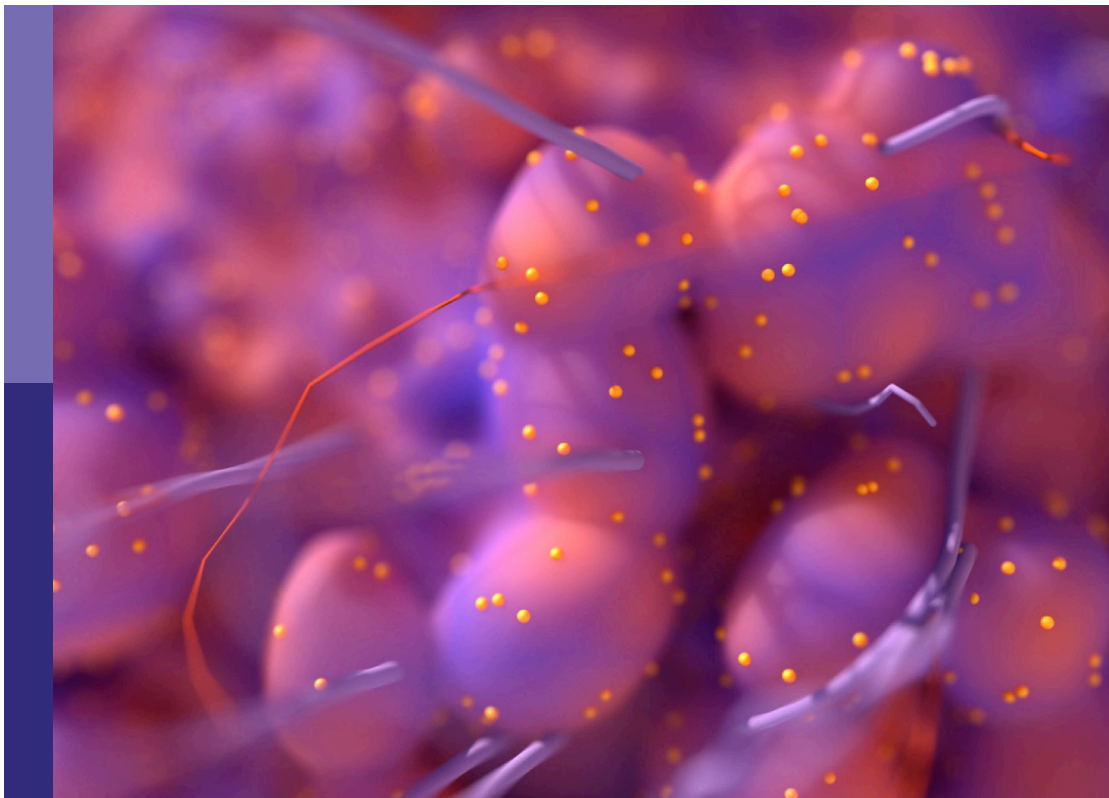
frontiers | Research Topics

Women in breast cancer 2022, volume II

Edited by
Margaret Gatti-Mays, Rachel Wuerstlein, Ariella Hanker and Svasti Haricharan

Published in
Frontiers in Oncology



ISSN 1664-8714
ISBN 978-2-8325-3834-0
DOI 10.3389/978-2-8325-3834-0

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Women in breast cancer: 2022, volume II

Topic editors

Margaret Gatti-Mays — The Ohio State University, United States
Rachel Wuerstlein — Ludwig Maximilian University of Munich, Germany
Ariella Hanker — University of Texas Southwestern Medical Center, United States
Svasti Haricharan — Sanford Burnham Prebys Medical Discovery Institute, United States

Citation

Gatti-Mays, M., Wuerstlein, R., Hanker, A., Haricharan, S., eds. (2023). *Women in breast cancer: 2022, volume II*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-3834-0

Table of contents

frontiers | Frontiers in Oncology

ORIGINAL RESEARCH
published: 07 June 2022
doi: 10.3389/fonc.2022.923467

SARS-CoV-2 M Protein Facilitates Malignant Transformation of Breast Cancer Cells

*Hoai-Nga Thi Nguyen, Marie Kawahara, Cat-Khanh Vuong, Mizuho Fukushige, Toshiharu Yamashita and Osamu Ohneda**

Graduate School of Comprehensive Human Science, Laboratory of Regenerative Medicine and Stem Cell Biology, University of Tsukuba, Tsukuba, Japan

Coronavirus disease 2019 (COVID-19) has spread faster due to the emergence of SARS-CoV-2 variants, which carry an increased risk of infecting patients with comorbidities, such as breast cancer. However, there are still few reports on the effects of SARS-CoV-2 infection on the progression of breast cancer, as well as the factors and mechanisms involved. In the present study, we investigated the impact of SARS-CoV-2 proteins on breast cancer cells (BCC). The results suggested that SARS-CoV-2 M protein induced the mobility, proliferation, stemness and *in vivo* metastasis of a triple-negative breast cancer (TNBC) cell line, MDA-MB-231, which are involved in the upregulation of NFκB and STAT3 pathways. In addition, compared to MDA-MB-231 cells, the hormone-dependent breast cancer cell line MCF-7 showed a less response to M protein, with the protein showing no effects of promoting proliferation, stemness, and *in vivo* metastasis. Of note, coculture with M protein-treated MDA-MB-231 cells significantly induced the migration, proliferation, and stemness of MCF-7 cells, which are involved in the upregulation of genes related to EMT and inflammatory cytokines. Therefore, SARS-CoV-2 infection might promote the ability of aggressive BCC to induce the malignant phenotypes of the other non-aggressive BCC. Taken together, these findings suggested an increased risk of poor outcomes in TNBC patients with a history of SARS-CoV-2 infection, which required a long-term follow-up. In addition, the inhibition of NFκB and STAT3 signaling pathways is considered as a promising candidate for the treatment of worsen clinical outcomes in TNBC patients with COVID-19.

Keywords: COVID-19, breast cancer, metastasis, EMT, SARS-CoV-2

OPEN ACCESS

Edited by:
Ariella Hanker, University of Texas Southwestern Medical Center, United States

Reviewed by:
Rajkumar KS, All India Institute of Medical Sciences, Rishikesh, India
Zongsheng He, Army Medical University, China

***Correspondence:**
Osamu Ohneda
oohneda@md.tsukuba.ac.jp

Specialty section:
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

Received: *19 April 2022*
Accepted: *16 May 2022*
Published: *07 June 2022*

Citation:
Nguyen H-NT, Kawahara M, Vuong C-K, Fukushige M, Yamashita T and Ohneda O (2022) SARS-CoV-2 M Protein Facilitates Malignant Transformation of Breast Cancer Cells. Front. Oncol. 12:923467. doi: 10.3389/fonc.2022.923467

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has spread globally since it was first detected in December 2019, resulting in a pandemic that has impacted most nations and resulted in about 250 million illnesses and 5 million deaths (1). COVID-19 is a severe infectious respiratory disease caused by SARS-CoV-2, a new highly contagious virus from the family coronaviridae, like SARS-CoV and MERS-CoV (2). After infection, SARS-CoV-2 causes acute powerful inflammation, which leads to a high mortality rate (2). Although urgent vaccination programs in many countries have helped

reduce the number of new cases and the severity of symptom in such cases, the long-term impact of SARS-CoV-2 infection on human health still needs to be investigated.

Among COVID-19 patients, COVID-19–infected cancer patients appear to be more vulnerable than others. Cancer patients have a higher risk of infection, more severe symptoms, and higher mortality rate than non-cancer patients (3). Previous reports have shown that cancer patients had a high incidental diagnosis of SAR2-CoV-2 infection and also suffered worse outcomes than the general population (4–10). Defects in their immune system due to chemotherapy and radiotherapy not only facilitate infection with SARS-CoV-2 (3) but also lead to difficulty enjoying the protective effects of COVID-19 vaccination (11). Furthermore, cancer-related chronic inflammation contributes to more severe symptoms when patients do get infected (3).

In cancer and COVID-19, inflammation is a common characteristic of the pathophysiology. Cytokine storm is a systemic hyperactive immune status characterized by massive cytokine release and is seen in both SARS-CoV-2 infection and cancer (12). Among the cytokines involved in cytokine storm, interleukin-6 (IL6) and tumor necrosis factor α (TNFα) are key proinflammatory cytokines, playing major driver roles in the acute immune response during severe COVID-19 infection (12). IL6 stimulates cancer cell activities and features, such as proliferation, mesenchymal transformation, metastasis, stemness and immune evasion (13, 14). Furthermore, SARS-CoV-2 infection-related cytokine storm and systemic inflammation can induce oxidative stress, DNA damage and genetic instability in normal cells, which trigger the development of benign tumors and malignant transformation in the presence of oncoviruses (15). In addition, Francescangeli et al. suggested that the prolonged inflammation, leukocyte hyperactivation, T-cell impairment and thrombocytosis associated with COVID-19 may create a suitable microenvironment to reawaken dormant cancer cells, particular those with stem-like characteristics that survive after chemotherapy or radiotherapy and have the potential to cause recurrence or metastasis (13, 14). Moreover, Wei et al. also reported a significant increase in serum cancer biomarkers levels in critical cases of COVID-19 disease (16), implying a correlation between SARS-CoV-2 infection and tumorigenesis. Therefore, studying the effects of COVID-19 on cancer is an urgent need in the field of preventive healthcare of cancer patients.

Breast cancer is one of the most frequent cancer types among SARS-CoV-2-infected cancer patients (6, 17, 18). Breast cancer patients infected with SARS-CoV-2 might develop new metastases, progression and death due to tumor progression (8, 9). Therefore, in the present study, we aimed to examine the correlation between SARS-CoV-2 infection and breast cancer progression by studying the effects of SARS-CoV-2 proteins on the phenotypes of different types of human breast cancer cells (BCC), including aggressive MDA-MB-231 cells and non-aggressive MCF-7 cells. In addition, the impact of SARS-CoV-2 proteins on the paracrine effects of aggressive BCC on non-aggressive BCC were also examined.

MATERIALS AND METHODS

Breast Cancer Cell Culture and Induction With SARS-CoV-2 Protein

The human breast cancer cell lines MDA-MB-231 (ATCC HTB-26) and MCF-7 (ATCC HTB-22) were cultured in Iscove's modified Dulbecco's (IMDM) medium (Gibco, Waltham, MA, USA) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained in humidified incubator with 5% CO_2 at 37°C. The medium was changed every two days. On reaching 80% confluence, the cells were trypsinized and subcultured at an initial density of 3.8×10^4cells/ml in culture medium.

A total of 5×10^5 cells/ml were then treated by SARS-CoV-2 peptivator Peptide Pools Prot_M (M protein; PepTivator SARS-CoV-2 Prot M, 130-126-702, Miltenyi Biotec, Cologne, Germany), SARS-CoV-2 peptivator Peptide Pools Prot_S (S protein; PepTivator SARS-CoV-2 Prot S, 130-126-700, Miltenyi Biotec) and SARS-CoV-2 peptivator Peptide Pools Prot_N (N protein; PepTivator SARS-CoV-2 Prot N, 130-126-698, Miltenyi Biotec) at concentrations of 60 pmol/ml for 24 h before further analyses.

The Migration Assay

MDA-MB-231 and MCF-7 cells were seeded into each well of a 24-well plate at 2×10^5 cells/400 µl/well and 1.5×10^5 cells/300 µl/well, respectively, in cultured medium and incubated for 1 hour before being treated with M protein, S protein and N protein. After 24 h of treatment, Mitomycin C solution (Nacalai Tesque, Kyoto, Japan) was added to the medium at 10 µg/ml for 2 h. A single scratch wound was then created using a 100-µl micropipette tip. The cultured medium was removed and changed to IMDM 0.25% FBS. Pictures were taken right after scratching and 24 h after scratching using a Keyence BZ-X710 microscope system (Keyence Corporation, Osaka, Japan). The migration distance (µm) at 0 and 24 h after wounding was measured using the ImageJ software program (NIH, Bethesda, MD, USA).

The Matrigel Invasion Assay

A suspension of MDA-MB-231 or MCF-7 cells (1×10^5 cells/200 µl) in IMDM medium was seeded onto a BD Matrigel Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ, USA)-coated 8-µm BD Falcon cell culture insert transwell (BD Biosciences). A total of 400 µl of IMDM supplemented with 10% FBS was added to the lower compartments of each chamber. The cells were treated with M protein for 24 h. After removing the cells remaining inside the transwell with a cotton swab, the bottom surface of each transwell membrane were then fixed in 4% paraformaldehyde in two minutes, permeabilized in methanol in 20 minutes. Then, the fixed transwell membranes were dipped in Hematoxylin in 30 seconds and washed in distilled water two times, following by dipping in Eosin in 20 seconds and washing in distilled water two times. The membranes were observed under microscope immediately after

staining. Five random pictures were taken for each transwell, and the average number of cells was counted using the ImageJ software program. The invasion rate was calculated as follows:

$$\text{Invasion percentage} = \text{Average number of cells in coated transwell} \times 100/\text{Average number of cells in uncoated transwell}$$

The Mammosphere Formation Assay

A suspension of MDA-MB-231 or MCF-7 cells (9.5×10^3 cells/2 ml) was mixed in MammoCult Basal medium (StemCell Technologies Inc., Vancouver, Canada) containing heparin and hydrocortisone and cultured for 5 days in a 6-well cell culture plate with an ultra-low-attachment surface (Corning; Corning, NY, USA). The mammosphere (diameter ≥100 μm)-forming efficiency (MSFE) was calculated as follows:

$$\text{MSFE}\ (\%) = \text{number of mammospheres} \times 100 \,/\, \text{number of seeded cells}$$

The Coculture Assay

MCF-7 cells were seeded into each well of a 24-well plate at 1×10^5 cells/200 μl/well in culture medium and incubated for 2 hours before coculture. A suspension of MDA-MB-231 or MCF-7 cells (1×10^5 cells/200 μl) seeded onto a 3-μm BD Falcon cell culture insert transwell (BD Biosciences) was then inserted into an MCF-7 cell-seeded 24-well plate. The cells were treated with M protein for 48 h before undergoing further examinations.

Collection of Conditioned Medium

MDA-MB-231 cells or MCF-7 at the density of 5×10^5 cells/ml were treated by M protein at concentrations of 60 pmol/ml for 24 h, then the conditioned medium was collected. The conditioned medium was centrifuged at 1000 rpm in 5 minutes, following by another step at 2000 rpm in 20 minutes to remove the cell death and cell debris. In order to inhibit the inflammatory cytokines, neutralizing antibodies were added to conditioned medium, including human anti-TNFα antibody (MAB210, R&D Systems, Minneapolis, MN, USA), anti-IL6 antibody (MAB2061, R&D Systems) and anti-IL8 antibody (MAB208, R&D Systems) at concentrations of 10 μg/ml, 5 μg/ml and 5 μg/ml respectively.

The Proliferation Assay

MDA-MB-231 and MCF-7 cells were seeded into each well of a 96-well plate at 1×10^4 cells/100 μl/well in culture medium and incubated for 1 hour before being treated with M protein. After 24, 48 and 72 h of treatment, the cell density was determined using a Cell Counting Kit (Dojindo Molecular Technologies, Kumamoto, Japan) 1 h before the absorbance was measured at a wavelength of 450 nm (OD_{450nm}).

Quantitative Reverse Transcription (qRT) PCR Gene Expression Analyses

To investigate the gene expression at the transcriptional level, qRT-PCR was carried out. MDA-MB-231 and MCF-7 cells were seeded into each well of a 6-well plate at 5×10^5 cells/1 ml/well in culture medium and incubated for 1 hour before being treated with M protein. After 24 hours, total RNA was isolated using Sepasol-RNA Super G (Nacalai Tesque) according to the instruction of the manufacturer. 2 μg of total RNA sample was reverse transcribed into cDNA using RT-PCR ReverTra Ace qPCR RT kit (Toyobo, Kita, Osaka, Japan). 2 μl of cDNA templates was subjected to realtime PCR amplification using THUNDERBIRD SYBR qPCR Mix (Toyobo) in Real-time PCR system QuantStudio 5 (Thermo Fisher Scientific). The qPCR program comprised an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation step at 95°C for 15s, annealing and extension step at 60°C for 30s. The sequences of qPCR primers for analysis were listed in **Table 1**. The expression levels of target genes were analyzed using the ΔΔCt method and normalized to the expression level of internal control housekeeping gene *ACTB* (β-actin) in each sample by the formula $2^{-\Delta Ct}$.

Western Blotting

MDA-MB-231 and MCF-7 cells were seeded into 10cm dish at 3.2×10^6 cells/6.4 ml/dish in culture medium and incubated for 1 h before being treated with M protein. After 24 h, cells were harvested, and nuclear proteins were extracted using RIPA buffer (25 mM Tris, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholic acid, 0.1% SDS) for 30 min; samples were then centrifuged at 15,000 rpm at 4°C for 10 min. The protein concentration was measured using the Bradford method (Biorad, Hercules, CA, USA).

Protein samples were denatured at 95°C for 3 minutes in sodium dodecyl sulfate (SDS) loading buffer (Wako Pure Chemical, Osaka, Japan) and subjected to SDS-polyacrylamide

TABLE 1 | Primer sequences.

Primer	Sequence
β-Actin	CTCGCCTTTGCCGATCC
	TCTCCATGTCGTCCCAGTTG
Vimentin	CCGTTGAAGCTGCTAACTACCAAGAC
	GTGGGTATCAACCAGAGGGAGTGAAT
N-Cadherin	GTGGAGGAGAAGAAGACCAGGACTATG
	CTAACAGGGAGTCATATGGTGGAGCTG
IL6	ACAAGAGTAACATGTGTGAAAGCAG
	TATACCTCAAACTCCAAAAGACCAG
IL8	GAGAGTGATTGAGAGTGGACCAC
	CACAACCCTCTGCACCCAGTTT
TNFα	TCCTTCAGACACCCTCAACC
	AGGCCCCAGTTTGAATTCTT
Snail	AACTACAGCGAGCTGCAGGACTCTAA
	CCTTTCCCACTGTCCTCATCTGACA
Twist	AGCCGCAGAGACCTAAACAA
	CACGCCCTGTTTCTTTGAAT
Slug	CTCCTCTTTCCGGATACTCCTCATCT
	CCAGGCTCACATATTCCTTGTCACAG
Zeb1	CAGCTCTGGGTGGAGAAGAC
	CCTGACCCACTTCCAACAGT
HIF-1α	TTACCGAATTGATGGGATATGAG
	TCATGATGAGTTTTGGTCAGATG
ACE2	AGGAGGTCTGAACATCATCAGTG
	GGGATCAGAGATCGGAAGAAGAAA
TMPRSS2	AATCGGTGTGTTCGCCTCTAC
	CGTAGTTCTCGTTCCAGTCGT

gel electrophoresis (PAGE) (50 μg per sample) and electrotransferred to PVDF membranes (Merck Millipore, Burlington, MA, USA). Membranes were then incubated with primary antibodies, including rabbit anti-pan-STAT3 (8204S; Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-phosphorylated STAT3 (pSTAT3) (8204S; Cell Signaling Technology) at 1:1000 dilution. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fischer Scientific) was used as a secondary antibody at 1:10000 dilution. Signals were detected by chemiluminescence HRP substrate (Merck Millipore) in an Image Quant LAS 4000 system (GE Healthcare, Chicago, IL, USA) and analyzed using the ImageJ software program.

The *In Vivo* Metastasis Assay

Female C57BL/6J mice were bred under specific-pathogen-free (SPF) conditions. All experimental procedures were approved by the University of Tsukuba Institute Animal Care and Use Committee. MDA-MB-231 and MCF-7 cells were seeded into each well of a 6-well plate at 5×10^5 cells/1 ml/well in culture medium and incubated for 1 hour before being treated with M protein. After 24 h, cells were harvested and resuspended in phosphate-buffered saline (PBS) before injection. The cell suspension (2×10^5 cells/300 μl) was injected into the tail vein, and mice were injected with Cyclosporin-A (Sigma-Aldrich) every day for the first week and every 2 days for the second week (200 μl per mouse) for immunosuppression.

After 14 days, the mice were sacrificed by cervical dislocation, and the lungs were collected, fixed with 4% paraformaldehyde (Wako Pure Chemical), and turned into frozen sections. The lung sections were stained by Hematoxylin–Eosin. All sections of each sample were observed under a microscope to find all tumors foci. The tumor foci area was measured by the ImageJ software program.

Statistical Analyses

The results were described as the mean ± standard deviation (SD). Differences were analyzed using the Mann Whitney U-test of the GraphPad Prism 5 software program (GraphPad Software Inc., San Diego, CA, USA). Differences were considered to be significant if P value of ≤0.05.

RESULTS

Different Responses of TNBC Cells and Hormone Dependent Cells to SARS-CoV-2 M Protein

Firstly we examined the effects of SARS-CoV-2 proteins, including membrane protein (M protein), spike protein (S protein) and nucleocapsid protein (N protein) on the migratory ability of aggressive breast cancer cells (BCC), MDA-MB-231 cells, and non-aggressive BCC, MCF-7 cells. The results showed that compared with S and N proteins, M protein shows a significantly greater ability to induce the migration of both MDA-MB-231 and MCF-7 cells (MDA-MB-231: 2.8-fold increase, MCF-7: 1.6-fold increase, **Figure 1A**). Therefore, we next examined the effects of M protein on the other phenotypes of BCC, including the invasion, proliferation, and stemness. As a result, M protein induced the invasion through Matrigel in both MDA-MB-231 and MCF-7 cells (MDA-MB-231 cells: 2.25-fold increase MCF-7 cells: 2.6-fold increase, **Figure 1B**). However, while M protein induced proliferation and sphere formation in MDA-MB-231 cells (proliferation: 1.4-fold increase after 72 h of treatment, sphere formation: 2.1-fold increase, **Figures 1D, E**), MCF-7 cells showed no marked induction of proliferation or stem-like sphere formation after treatment with M protein (**Figures 1C, D**).

We next examined the effects of M protein on the *in vivo* metastasis of BCC in a lung metastatic mouse model. BCC (including those treated by M protein) were intravenously injected into mice *via* the tail vein, and then the number of tumor foci and the size of tumors in the lungs were examined (**Figure 1E**). As a result, mice injected with M protein-treated MDA-MB-231 cells showed significantly more tumor foci in the lungs (2.5-fold increased, **Figure 1F**) than those injected with untreated MDA-MB-231 cells. In addition, M protein-treated MDA-MB-231 cells tended to form larger tumors with a greater tumor size variability in mouse lungs than untreated MDA-MB-231 cells (**Figure 1G**). In contrast to MDA-MB-231 cells, MCF-7 cells showed no induced metastatic ability *in vivo* after treatment with M protein. Mice injected with M protein-treated MCF-7 cells showed no significant difference in the number of tumor foci (**Figures 1E, F**) from those injected with untreated MCF-7 cells, and although there were some remarkably large tumors (>0.4 mm^2) in mice injected with M protein-treated MCF-7 cells, the median tumor size did not remarkably increase compared to those injected with untreated MCF-7 cells (**Figure 1G**).

Epithelial to mesenchymal transition (EMT) plays an important role in tumor progression and metastasis (19). Upon EMT, carcinoma cells lose epithelial marker expression and cell polarity and instead acquire the mesenchymal morphology, mobility and invasion capabilities critical for tumor invasion and metastasis (19). In addition, cancer cells obtain stem cell-like characteristics through EMT, which facilitates cancer relapse and metastasis (20). Therefore, we next examined the effects of M protein on the expression of genes related to EMT, proliferation and stemness of BCC. The results showed that M protein upregulated the expression of genes related to stemness and EMT, such as Twist (5.4-fold increase), Zeb1 (16-fold increase), HIF-1α (1.5-fold increase) and Snail (7.2-fold increase) in MDA-MB-231 cells, and Twist (3.9-fold increase), Zeb1 (3.7-fold increase), HIF-1α (2.5-fold increase) and Slug (2.7-fold increase) in MCF-7 cells (**Figure 1H**). In addition, while MDA-MB-231 cells treated with M protein showed an increased expression of mesenchymal markers, including N-Cadherin (2-fold increase) and Vimentin (3.5-fold increase) (19), MCF-7 cells showed no altered expression of these genes by M protein (**Figure 1I**). Interestingly, the expressions of ACE2 and TMPRSS2, the binding receptors of SARS-CoV-2, were

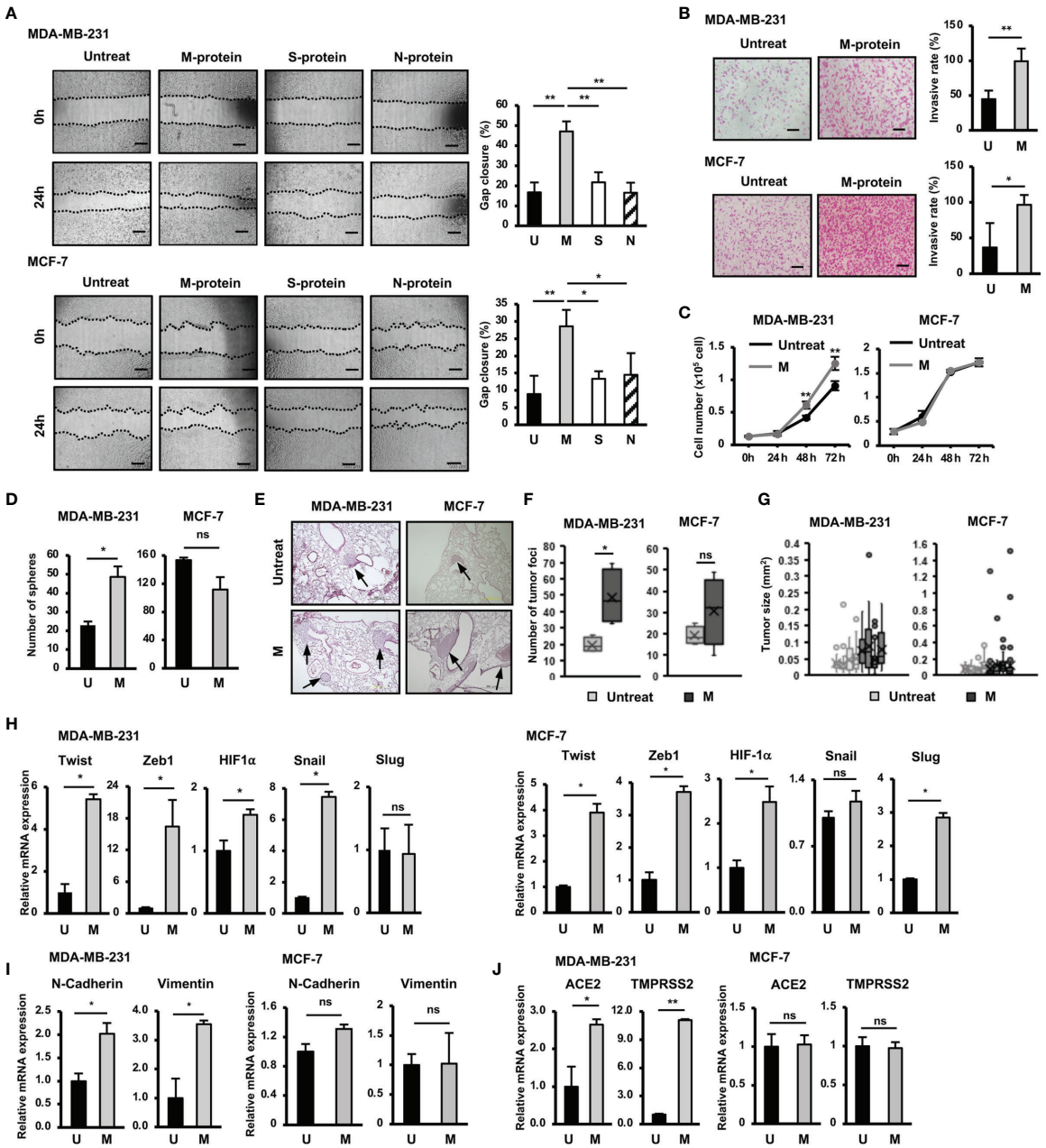


FIGURE 1 | Responses of BCC to SARS-CoV-2 protein. **(A)** The migration of BCC in response to SARS-CoV-2 proteins. **(B)** The BCC invasion assay in response to M protein. **(C)** The proliferation of BCC in response to M protein. **(D)** The mammosphere assay of BCC in response to M protein. **(E)** Lung sections from an *in vivo* metastasis assay of BCC, arrows indicate tumors in lungs. **(F)** Number of tumor foci from an *in vivo* metastasis assay. **(G)** Tumor sizes from an *in vivo* metastasis assay in individual mice; each dot indicates a tumor. **(H)** The mRNA expression of genes related to EMT process in BCC. **(I)** The mRNA expression of EMT markers in BCC. **(J)** The mRNA expression of SARS-CoV-2 binding receptor genes in BCC. U, untreated BCC; M, M protein-treated BCC. The scale bars indicate 500 μm. Each value represents the mean ± SD of triplicate experiments. (ns, no significance; $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$).

upregulated by the induction of M protein in MDA-MB-231 cells (ACE2: 2.8-fold increase, TMPRSS2: 11.3-fold increase) but not in MCF-7 cells (**Figure 1J**).

Taken together, these data suggested different responses to SARS-CoV-2 M protein between TNBC cells and hormone-dependent BCC. While M protein induced the *in vitro* migration and invasion of both MDA-MB-231 and MCF-7 cells, only MDA-MB-231 cells showed the promotion of proliferation, stemness and *in vivo* metastasis in response to M protein.

Involvement of the NFκB Pathway in the Induction of the Upregulation of EMT Genes and Migration of BCC by SARS-CoV-2 M Protein

SARS-CoV-2 infection is associated with cytokine storm characterized by the massive release of inflammatory cytokines, including IL6, TNFα and IL8 (12, 21). In addition, previous studies have suggested a relationship between inflammatory cytokines and EMT in cancer cells (22). Therefore, we next examined the expression of inflammatory cytokines treated with M protein in BCC. As shown in **Figure 2A**, after treatment with M protein, MDA-MB-231 cells showed the significant upregulation of TNFα, IL6 and IL8 (TNFα: 6.6-fold increase, IL6: 9.4-fold increase; IL8: 2.6-fold increase). However, M protein showed no effect of inducing the expression of these inflammatory cytokines in MCF-7 cells (**Figure 2A**).

Numerous studies have reported the role of the NFκB signaling pathway in the upregulation of inflammatory

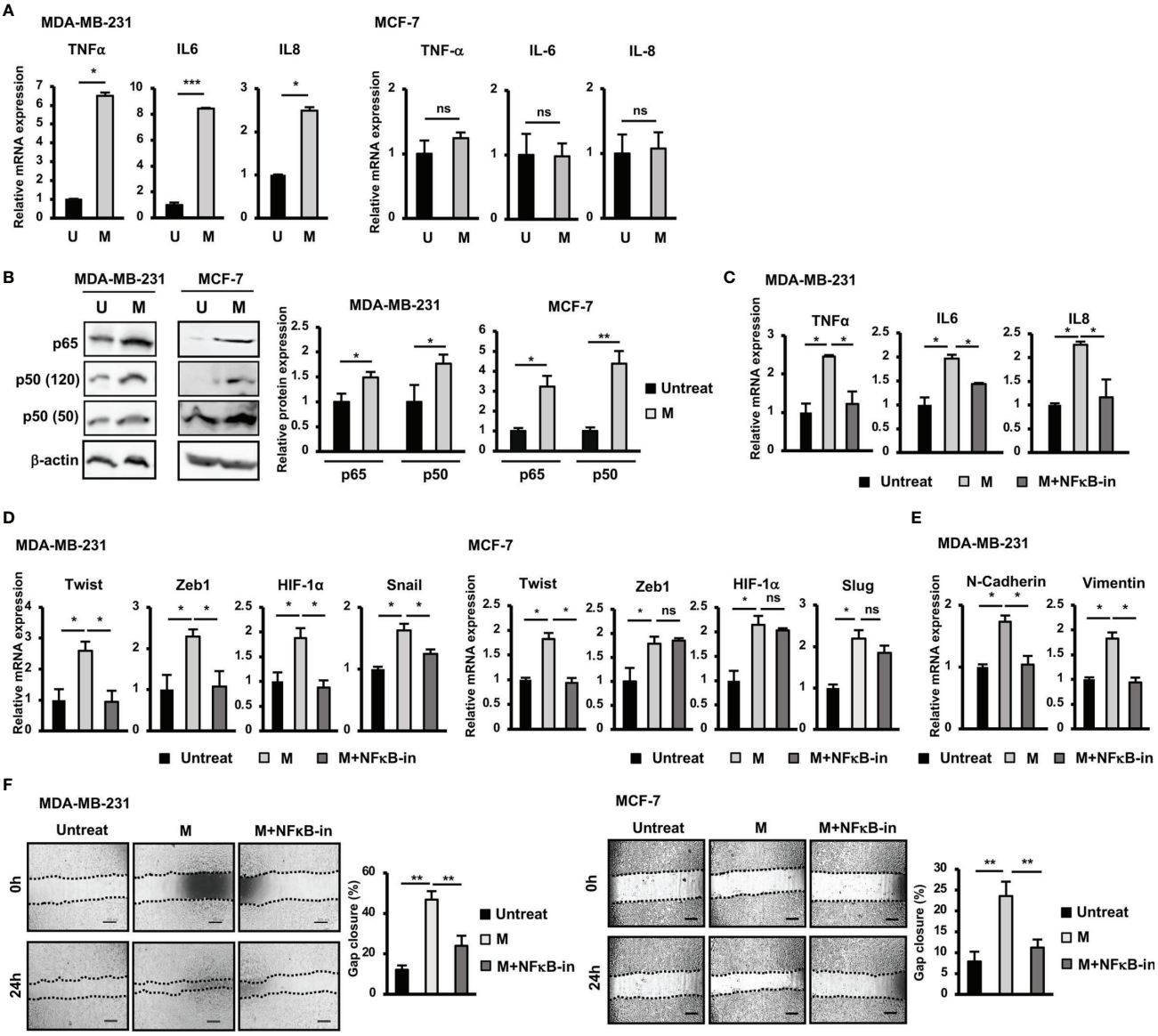


FIGURE 2 | NFκB pathway was involved in the upregulation of EMT genes and the induction of migration of BCC by SARS-CoV-2 M protein. **(A)** Gene expression of inflammatory cytokines in BCC treated with M protein. **(B)** Protein expression of p65 and p50 in BCC treated with M protein. **(C)** The mRNA expression of inflammatory cytokines in BCC in the presence of M protein and NFκB inhibitor. **(D)** The mRNA expression of genes related to EMT process in BCC in the presence of M protein and NFκB inhibitor. **(E)** The mRNA expression of EMT markers in MDA-MB-231 cells treated with M protein in the presence of NFκB inhibitor. **(F)** Migration of BCC treated with M protein in the presence of NFκB inhibitor. BAY11-7082 10 μM was used as NFκB inhibitor. U, untreated BCC; M, M protein-induced BCC. The scale bars indicate 500 μm. Each value represents the mean ± SD of triplicate experiments. (ns, no significance; $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

cytokines, such as IL6, IL8, and TNFα (23). In addition, NFκB activation in breast cancers results in EMT and a stem-like phenotype (23). Therefore, we next examined the involvement of NFκB signaling pathway in the upregulation of inflammatory and EMT genes in M protein-treated BCC. As shown in **Figure 2B**, both MDA-MB-231 and MCF-7 cells showed an increase in p50 (MDA-MB-231: 1.8-fold increase, MCF-7: 3.2-fold increase) and p65 (MDA-MB-231: 1.5-fold increase, MCF-7: 4.4-fold increase) in response to M protein. Of note, in the presence of an NFκB inhibitor, M protein-treated MDA-MB-231 cells showed the downregulation of inflammatory cytokines (**Figure 2C**).

Next, we examined the effect of treatment with an NFκB inhibitor on the expression of EMT genes which were upregulated by M protein in MDA-MB-231 and MCF-7 cells. As shown in **Figure 2D**, treatment with an NFκB inhibitor impaired the expression of EMT genes upregulated by M protein in MDA-MB-231, such as Twist (2.2-fold decrease), Zeb1 (2.5-fold decrease), HIF-1α (2-fold decrease) and Snail (1.4-fold decrease). However, while treatment with an NFκB inhibitor induced the downregulation of Twist expression (2-fold decrease) in M protein-treated MCF-7 cells, no effects on the other genes, such as Zeb1, Slug or HIF-1α, were observed (**Figure 2D**). In addition, treatment with an NFκB inhibitor resulted in the downregulation of mesenchymal markers, including N-Cadherin (1.7-fold decrease) and Vimentin (1.8-fold decrease) in M protein-treated MDA-MB-231 (**Figure 2E**). We next examined the effects of NFκB inhibitor on the migration of M protein-treated BCC. As a result, treatment with NFκB inhibitor significantly decreased the migratory ability of both M protein-treated MDA-MB-231 cells and M protein-treated MCF-7 cells (MDA-MB-231: 2-fold decrease, MCF-7: 2.1-fold decrease, **Figure 2F**).

Taken together, these results suggested that M protein significantly induced the expression of inflammatory cytokines in MDA-MB-231 cells but not MCF-7 cells, which was involved in the upregulation of the NFκB pathway. Of note, the NFκB pathway was also highly involved in the M protein-induced migration of both MDA-MB-231 and MCF-7 cells.

Contribution of STAT3 Pathway Activation to the Induction of the Upregulation of EMT Genes and Migration of BCC by SARS-CoV-2 M Protein

In addition to the NFκB pathway, the Jak/STAT3 pathway is also reportedly involved in the upregulation of genes related to EMT in BCC (24). Therefore, we examined the role of the STAT3 pathway in the responses of BCC to M protein. The results showed that treatment with M protein significantly activated the phosphorylation of STAT3 in both MDA-MB-231 and MCF-7 cells (MDA-MB-231: 2.3-fold increase, MCF-7: 3-fold increase, **Figure 3A**).

In addition, while treatment with a STAT3 inhibitor showed no marked effects on the expression of inflammatory cytokines in MDA-MB-231 cells (**Figure 3B**), it resulted in the reduced expression of EMT genes upregulated by M protein, such as Twist (2.2-fold decrease), Zeb1 (7.4-fold decrease), HIF-1α (1.95-fold decrease), Snail (1.5-fold decrease) in MDA-MB-231 cells and Zeb1 (1.7-fold decrease) in MCF-7 cells (**Figure 3C**). In addition, treatment with a STAT3 inhibitor impaired the expression of mesenchymal markers, such as N-Cadherin (2.4-fold decrease), and Vimentin (2.9-fold decrease) in M protein-treated MDA-MB-231 cells (**Figure 3D**).

Next, we examined the role of the STAT3 pathway in the induced migratory ability of BCC by M protein. As a result, treatment with a STAT3 inhibitor significantly suppressed M protein-induced migration in both MDA-MB-231 and MCF-7 cells (MDA-MB-231: 2.9-fold decrease, MCF-7: 1.5-fold decrease, **Figure 3E**).

To examine the relationship between the NFκB and STAT3 pathways in M protein-treated MDA-MB-231 cells, we next examined the phosphorylation of STAT3 protein in M protein-treated MDA-MB-231 cells cultured in the presence of an NFκB inhibitor. As shown in **Figure 3F**, an NFκB inhibitor suppressed the phosphorylation of STAT3 in M protein-treated MDA-MB-231 cells (1.3-fold decrease, $p<0.05$), suggesting that the activation of the NFκB pathway might trigger the STAT3 pathway in M protein-treated MDA-MB-231 cells.

Taken together, these data suggested that, in MDA-MB-231 cells, M protein activated NFκB, consequently upregulating inflammatory cytokines and the STAT3 pathway, which are involved in the induction of EMT and migration. However, in MCF-7 cells, the activation of both the NFκB and STAT3 pathways was involved in the induction of the expression of EMT genes and migration.

Promotion of Mobility, Proliferation and Stemness of MCF-7 Cells by SARS-CoV-2 M Protein-Treated MDA-MB-231 Cells

Tumors are a heterogenous mixture of different malignant and nonmalignant cells, in which non-aggressive cells can acquire new phenotypes through communication with aggressive cells, promoting malignancy (25). Therefore, to examine the effects of aggressive BCC on non-aggressive BCC, we next cocultured MCF-7 cells, a non-aggressive BCC line, with MDA-MB-231 cells, an aggressive BCC line, and characterized the altered phenotypes of MCF-7 cells. The results showed that coculturing with MDA-MB-231 cells significantly induced the proliferation of MCF-7 cells (1.3-fold increase after 72 h coculturing, **Figure 4A**) but showed no effects on the sphere formation or migration of MCF-7 cells (**Figures 4B, C**). We then examined the effects of MDA-MB-231 cells on the gene expression of MCF-7 cells. Coculturing with MDA-MB-231 cells significantly induced the expression of Snail (1.4-fold increase) but showed no effects on the expression of the other EMT genes (**Figure 4D**). In addition, coculturing with MDA-MB-231 cells upregulated the expression of Vimentin (1.7-fold increase, **Figure 4E**), a mesenchymal marker in EMT in MCF-7 cells. However, coculturing with MDA-MB-231 cells showed no effects on the expression of inflammatory cytokines, including TNFα, IL6 and IL8 in MCF-7 cells (**Figure 4F**). Interestingly, coculturing with MDA-MB-231 cells significantly upregulated

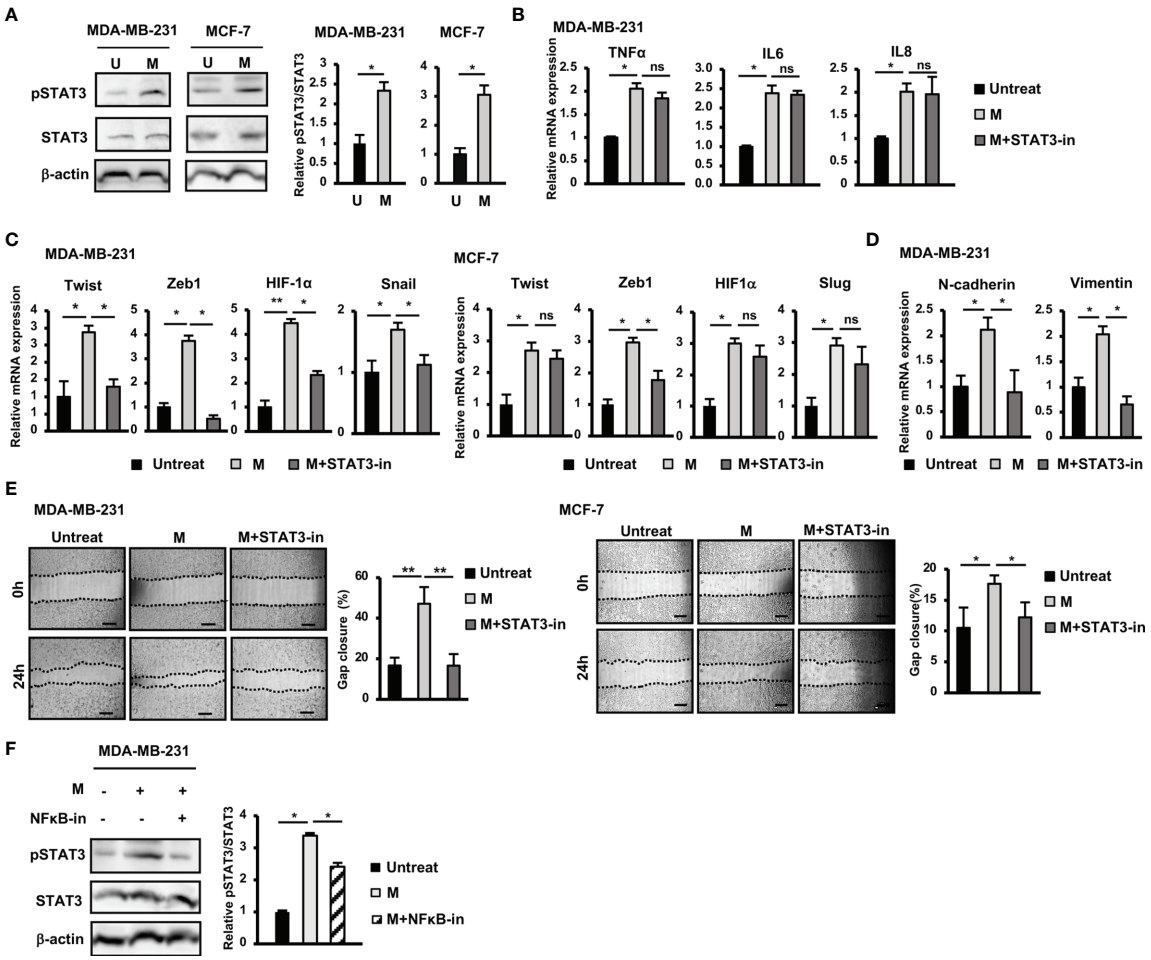


FIGURE 3 | Activation of STAT3 pathway contributed to the upregulation of EMT genes and the induction of migration of BCC by SARS-CoV-2 M protein. **(A)** The phosphorylation of STAT3 in BCC treated with M protein. **(B)** Gene expression of inflammatory cytokines in MDA-MB-231 cells treated with M protein in the presence of STAT3 inhibitor. **(C)** EMT-related gene expression in BCC treated with M protein in the presence of STAT3 inhibitor. **(D)** The mRNA expression of EMT markers in MDA-MB-231 cells treated with M protein in the presence of STAT3 inhibitor. **(E)** Migration of BCC treated with M protein in the presence of STAT3 inhibitor. **(F)** The phosphorylation of STAT3 in MDA-MB-231 in the presence of M protein and the NFκB inhibitor. Galiellalactone 100 ng/ml (SC-202165; Santa Cruz Biotechnology) was used as a STAT3 inhibitor. BAY11-7082 10 μM was used as an NFκB inhibitor. U, untreated BCC; M, M protein-induced BCC. The scale bars indicate 500 μm. Each value represents the mean ± SD of triplicate experiments. (ns, no significance; $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$).

the expression of ACE2 (2.74-fold increase, **Figure 4G**), a binding receptor of SARS-CoV-2.

Next, we examined the effects of M-protein on the ability of MDA-MB-231 cells to alter the phenotypes of MCF-7 cells. First, MCF-7 cells were cocultured with M protein-treated MDA-MB-231 cells, and then the gene expression was compared with that of MCF-7 cells treated with M protein directly. The results showed that coculturing with M protein-treated MDA-MB-231 cells induced the expression of ACE2, the SARS-CoV-2-binding receptor, in MCF-7 cells (3.6-fold increase, **Figure 4H**). Of note, coculturing with M protein-treated MDA-MB-231 cells upregulated the expression of inflammatory cytokines, which was not seen in MCF-7 cells treated with M protein directly (IL6: 2-fold increase, IL8: 5.3-fold increase, and TNFα:1.6-fold increased, **Figure 4I**). In addition, M protein-treated MDA-MB-231 cells significantly induced the expression of Vimentin (2.2-fold increase, **Figure 4J**), but showed no effects to upregulated other genes related to EMT in MCF-7 cells (**Figure 4K**).

We then examined the effects of M protein-treated MDA-MB-231 cells on the phenotypes of MCF-7 cells in comparison to MCF-7 cells treated with M protein directly. The results showed that M protein-treated MDA-MB-231 cells significantly induced the proliferation of MCF-7 cells (1.7-fold increase after 96 h of coculturing, **Figure 4L**). Notably, M protein-treated MDA-MB-231 cells showed the significant promotion of sphere formation (1.64-fold increase, **Figure 4M**) and migration (1.43-fold increase, **Figure 4N**) of MCF-7 cells, findings that were not observed in MCF-7 cells cocultured with MDA-MB-231 cells.

M protein-treated MDA-MB-231 cells showed the upregulation of inflammatory cytokines such as TNFα, IL6 and IL8 (**Figure 2A**), which were reported to be involved in

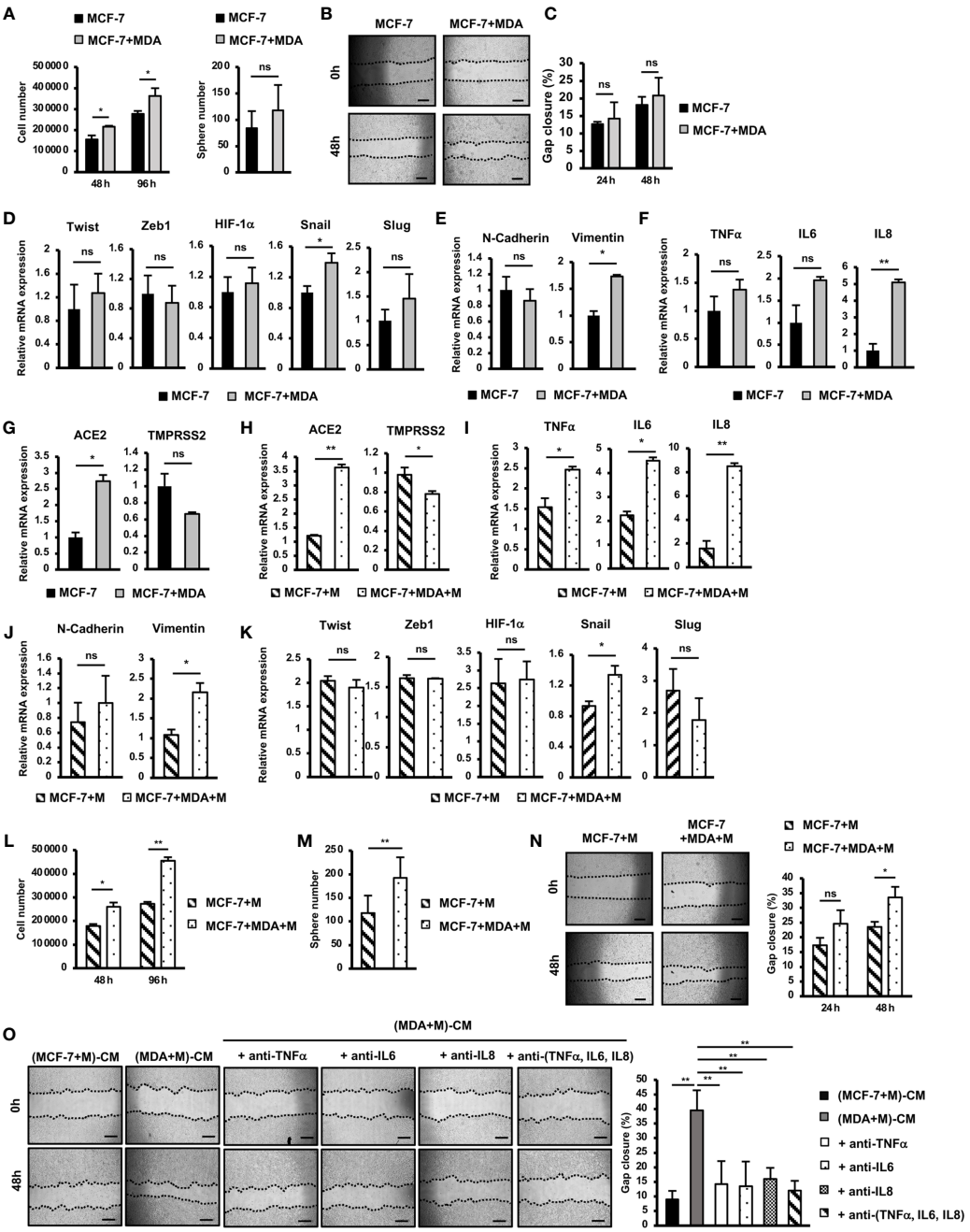


FIGURE 4 | SARS-CoV-2 M protein-treated MDA-MB-231 cells promoted the mobility, proliferation and stemness of MCF-7 cells. **(A)** Proliferation of MCF-7 cells cocultured with MDA-MB-231 cells. **(B)** A mammosphere assay of MCF-7 cells cocultured with MDA-MB-231 cells. **(C)** Migration of MCF-7 cells cocultured with MDA-MB-231 cells. **(D)** The mRNA expression of genes related to EMT process in MCF-7 cells cocultured with MDA-MB-231 cells. **(E)** The mRNA expression of EMT markers in MCF-7 cells cocultured with MDA-MB-231 cells. **(F)** Gene expression of inflammatory cytokines in MCF-7 cells cocultured with MDA-MB-231 cells. **(G)** Gene expression of SARS-CoV-2 binding receptors in MCF-7 cells cocultured with MDA-MB-231 cells. **(H)** Gene expression of SARS-CoV-2 binding receptors in MCF-7 cells cocultured with M protein-treated MDA-MB-231 cells. **(I)** Gene expression of inflammatory cytokines in MCF-7 cells cocultured with M protein-treated MDA-MB-231 cells. **(J)** Gene expression of EMT markers in MCF-7 cells cocultured with M protein-treated MDA-MB-231 cells. **(K)** The mRNA expression of genes related to EMT process in MCF-7 cells cocultured with M protein-treated MDA-MB-231 cells. **(L)** Proliferation of MCF-7 cells cocultured with M protein-treated MDA-MB-231 cells. **(M)** Mammosphere assay of MCF-7 cells cocultured with M protein-treated MDA-MB-231 cells. **(N)** Migration of MCF-7 cells cocultured with M protein-treated MDA-MB-231 cells. **(O)** Migration of MCF-7 cells in conditioned medium (CM) from M protein-treated MCF7 cells or M protein-treated MDA-MB-231 cells in the presence of anti-TNFα antibody, anti-IL6 antibody and anti-IL8 antibody. The scale bars indicate 500 μm. Each value represents the mean ± SD of triplicate experiments. (ns, no significance; $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$).

the EMT and metastasis of BCC (26, 27). Therefore, we speculated the effects of these cytokines on the induced migration of MCF-7 cells by M protein-treated MDA-MB-231 cells. To check this hypothesis, the migration of MCF-7 cells in the conditioned medium-derived from M protein-treated MDA-MB-231 cells (CM) were compared with those in CM with the addition of neutralizing antibodies for TNFα, IL6, and IL8. As shown in **Figure 4O**, while CM significantly promoted the migration of MCF-7 cells, CM with the addition of neutralizing antibodies, either individually or together, showed no induced effects on the migration of MCF-7 cells. These data suggested that the upregulation of TNFα, IL6 and IL8 contributed to the induced paracrine effects of M protein-treated MDA-MB-231 cells on the migration of MCF-7 cells.

Taken together, these data suggested that, in addition to promoting the metastatic phenotypes of MDA-MB-231 cells, M protein also induces the paracrine effects of MDA-MB-231 cells on other non-aggressive BCC, thereby facilitating cancer progression. Specifically, M protein-treated MDA-MB-231 cells induced migration, proliferation and stemness, which might be involved in the upregulation of inflammatory cytokines and EMT genes, of MCF-7 cells.

DISCUSSION

Numerous studies have suggested that, in addition to having a high risk of SARS-CoV-2 infection, cancer patients might have an increased risk of accelerated cancer progression following infection (6–10). In addition, several case reports showed that breast cancer developed worsened outcomes after being infected by SARS-CoV-2, including new metastases and death due to tumor progression (8, 9). In the present study, our results demonstrated that SARS-CoV-2 M protein stimulated the migration, invasion and expression of EMT genes in both MDA-MB-231 cells, a TNBC cell line, and MCF-7 cells, a hormone-dependent BCC line (28, 29). These results were in line with those of a previous study in which sera from COVID-19 patients induced EMT and Vimentin, Zeb1 and Snail expression in lung, breast and colon cancer cells *in vitro* (10).

Previous study suggested that breast tumor tissues from TNBC patients showed the expression of ACE2, a receptor of SARS-CoV-2 (30). In the present study, we found that while MCF-7 cells showed the low expression of ACE2 and TMPRSS2 on the cell membrane surfaces, MDA-MD-231 cells exhibited the high expression of these receptors (**Supplementary Figure 1**). Among subtypes of breast cancer, TNBC is an aggressive type with a poor prognosis and low efficacy of targeted treatment (31). This raises concerns that cancer progression might be exacerbated when TNBC patients are infected with SARS-CoV-2. Of note, our finding suggested that MDA-MB-231 cells, but not MCF-7 cells, showed the induced aggressive phenotypes, including proliferation, stemness and *in vivo* metastasis by M protein. Therefore, it is necessary to perform further studies with a long-term follow-up of TNBC patients after SARS-CoV-2 infection.

In BCC, the activation of NFκB, a proinflammatory transcription factor, drives the inflammatory responses, proliferation, migration and invasion, leading to cancer development and progression. NFκB is also involved in the expansion of breast cancer stem cells, which are intimately associated with cancer relapse and metastasis. In clinical studies, the enhanced activation of NFκB is associated with the breast tumor size, malignant progression, aggressive behavior and metastases in breast cancer (23). A previous study reported that M protein of coronaviruses triggered the NFκB signaling pathway in MDA-MB-231 cells (32). Consistently, our study showed that M protein of SARS-CoV-2 activated the NFκB pathway, which is responsible for the upregulation of EMT and tumor progression-related genes, such as Zeb1/2, Snail, Twist and HIF-1α, in MDA-MB-231 cells (33). In addition, the activation of the NFκB pathway in M protein-treated MDA-MB-231 cells also induced the expression of inflammatory cytokines, including IL6, IL8, and TNFα, which are involved in tumor initiation and homing and metastasis of BCC (34–36) and might contribute to the amplification of the cytokine storm.

Numerous studies reported that STAT3, a signaling pathway associated with migration, invasion and cell plasticity, is associated with the NFκB pathway and stem-like phenotype of BCC (24, 37, 38). In addition, STAT3 was reported to be activated by inflammatory cytokines, such as IL6, IL8 and TNFα, which enhance breast cancer proliferation, invasion and metastasis through the upregulation of Twist, Snail, Slug, Vimentin and HIF-1α (24). In the present study, our findings suggested crosstalk between the NFκB and Jak/STAT3 signaling pathways through the autocrine expression of IL6, IL8 and TNFα in MDA-MB-231 cells induced by SARS-CoV-2 M protein. Therefore, the NFκB and Jak/STAT3 signaling pathways might be promising targets of treatment for TNBC patients who develop COVID-19 infection.

Tumors are a heterogenous mixture of cancer cells, in which non-aggressive cells can acquire new phenotypes such as malignancy through communication with aggressive cells (25). By coculturing of MCF-7, as a non-aggressive BCC, with MDA-MB-231, as an aggressive BCC, we found that MDA-MB-231 cells induced the proliferation, migration, and the expression of Vimentin, a mesenchymal marker, of MCF-7 cells. Our data are in line with those of previous studies which suggested that TNBC cells induce other subtypes of BCC to transform to an aggressive phenotype (39, 40). Of note, our findings suggested that M protein induction amplified the ability of MDA-MB-231 cells to induce the transition to an aggressive phenotype of MCF-7 cells, including the migration, proliferation, stemness and inflammatory cytokine expression, which were not happened in MCF-7 cells directly treated by M protein (**Figure 5**). Interestingly, our findings showed that the coculture of MCF-7 cells with M protein-treated MDA-MB-231 cells significantly induced the expression of ACE2 in MCF-7 cells. As ACE2 also serves as a biomarker of EMT and metastasis (15, 41), these data hinted that the upregulation of ACE2 by M protein-induced aggresstive BCC might facilitate the infection of SARS-CoV-2 and metastasis in non-aggressive BCC; suggesting that, in the

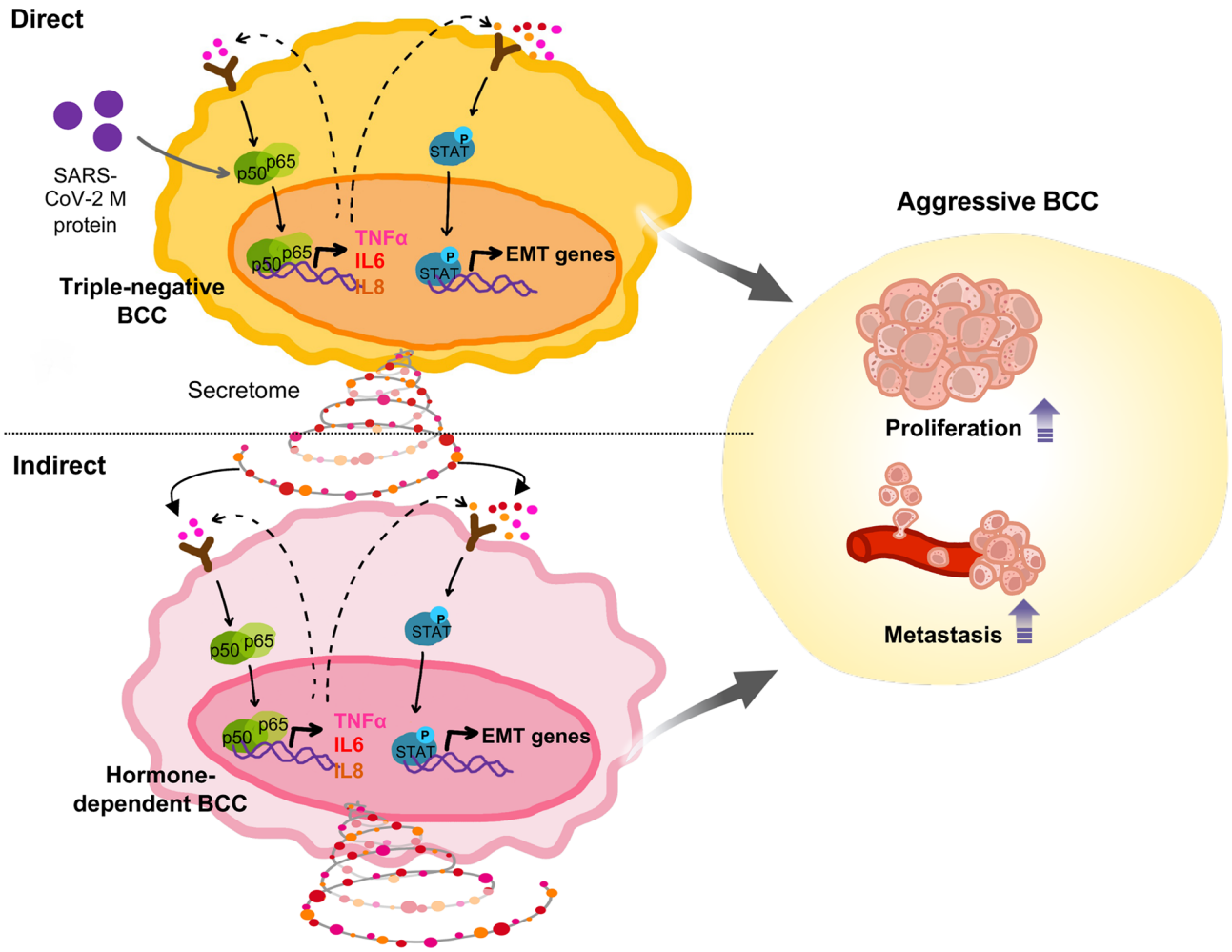


FIGURE 5 | Proposed model: In TNBC cells, SARS-CoV-2 M protein activated the NFκB and STAT3 signaling pathways, which are involved in mobility, proliferation and stemness, thereby facilitating *in vivo* metastasis. In addition, SARS-CoV-2 M protein-treated TNBC cells caused malignant transformation of non-aggressive BCC.

heterogenous mixture of cells inside tumors, SARS-CoV-2-infected aggressive BCC may affect non-aggressive BCC through secretome and cytokine storm and promote a poor general outcome of tumor progression. Our present data were based on established cell lines instead of patient samples; therefore, it is worth to investigate the effects of SARS-CoV-2 infection on the interaction between aggressive BCC and non-aggressive BCC derived from breast cancer patients.

Numerous reports showed the existence of SARS-CoV-2 viral proteins in sera of COVID-19 patients (42–45), suggesting that in addition to the direct infection of SARS-CoV-2 into cells, free viral proteins of SARS-CoV-2 in sera might also affect the surrounding cells. However, although several studies reported the effects of SARS-CoV-2 proteins on numerous types of cells (46–48), how SARS-CoV-2 protein, such as M protein, gets internalized into the cell is still obscured. Therefore, it is noteworthy for a further study to examine whether M protein binds to a specific receptor in the membrane surface of breast cancer cells or is non-selectively internalized into cells through macropinocytosis (49).

CONCLUSION

In summary, the present study demonstrated the effects of SARS-CoV-2 M protein on the malignant phenotypes of TNBC MDA-MB-231 cells, including the invasion, proliferation, stemness and *in vivo* metastasis of TNBC MDA-MB-231 cells, which might be involved in the upregulation of EMT genes regulated by the NFκB and Jak/STAT3 signaling pathways. Of note, M protein promoted the ability of MDA-MB-231 cells to induce malignant phenotypes in nonaggressive BCC lines, such as the hormone-dependent line MCF-7. Therefore, our findings suggested an increased risk of poor outcomes in breast cancer patients following SARS-CoV-2 infection, which should be noted while caring for cancer patients with COVID-19.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Care Committee of the University of Tsukuba.

AUTHOR CONTRIBUTIONS

H-NN contributed to the study concept, conducted the experiments and data analysis, and wrote the original draft of manuscript. MK contributed to the experiments, interpretation and data analysis. MF and C-KV contributed to the study concept, writing and editing of the manuscript. TY contributed to the *in vivo* experiments and technical support. OO raised the study concept and design, editing of the manuscript and final approval. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We would like to acknowledge the support from the Japanese Ministry of Education, Culture, Sports, Science & Technology (MEXT).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.923467/full#supplementary-material

Supplementary Figure 1 | MDA-MB-231 cells showed higher expression of SARS-CoV2 binding receptors, ACE2 and TMPRSS2, in comparison to MCF-7 cells. BCC were stained with primary antibodies, including rabbit Anti-ACE2 antibody (GTX101395, Genetex, Irvine, CA, USA) or rabbit Anti-TMPRSS2 antibody (GTX81494, Genetex) following by secondary antibody Goat Anti-rabbit Igg DyLight488 (GTX213110-04, Genetex). The expression of ACE2 and TMPRSS2 was examined by fluorescence activated cell sorting (FACS) analysis.

Supplementary Figure 2 | Full-length blots of Western blot figures. (A) Protein expression of p65 and p50 in MDA-MB-231 and MCF7 cells, which shown in Figure 2B. (B) The phosphorylation of STAT3 protein in MDA-MB-231 and MCF7 cells, which shown in Figure 3A. (C) The phosphorylation of STAT3 protein in MDA-MB-231 and MCF7 cells in the present of NFκB inhibitor, which shown in Figure 3F.

REFERENCES

1. World Health Organization (WHO). WHO Coronavirus (COVID-19) Dashboard, in: *World Health Organization (WHO)*. Available at: https://covid19.who.int/table (Accessed 10th November 2021).
2. Hu B, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. *Nat Rev Microbiol* (2021) 19(3):141–54. doi: 10.1038/s41579-020-00459-7
3. Derosa L, Melenotte C, Griscelli F, Gachot B, Marabelle A, Kroemer G, et al. The Immuno-Oncological Challenge of COVID-19. *Nat Cancer* (2020) 1 (10):946–64. doi: 10.1038/s43018-020-00122-3
4. Yang L, Chai P, Yu J, Fan X. Effects of Cancer on Patients With COVID-19: A Systematic Review and Meta-Analysis of 63,019 Participants. *Cancer Biol Med* (2021) 18(1):298–307. doi: 10.20892/j.issn.2095-3941.2020.0559
5. Alessandro A, Gangemi S, Pioggia G, Tonacci A, Musolino C. Cancer and SARS-CoV-2 Infection: Diagnostic and Therapeutic Challenges. *Cancers* (2020) 12(6):1581. doi: 10.3390/cancers12061581
6. Bertuzzi AF, Marrari A, Gennaro N, Cariboni U, Ciccarelli M, Giordano L, et al. Low Incidence of SARS-Cov-2 in Patients With Solid Tumours on Active Treatment: An Observational Study at a Tertiary Cancer Centre in Lombardy, Italy. *Cancers* (2020) 12(9):1–9. doi: 10.3390/cancers12092352
7. de Joode K, Oostvogels AAM, GeurtsvanKessel CH, de Vries RD, Mathijssen RHJ, Debets R, et al. Case Report: Adequate T and B Cell Responses in a SARS-CoV-2 Infected Patient After Immune Checkpoint Inhibition. *Front Immunol* (2021) 12:627186. doi: 10.3389/fimmu.2021.627186
8. Marenco-Hillembrand L, Erben Y, Suarez-Meade P, Franco-Mesa C, Sherman W, Eidelman BH, et al. Outcomes and Surgical Considerations for Neurosurgical Patients Hospitalized With COVID-19–A Multicenter Case Series. *World NeurosurG* (2021) 154:e118–29. doi: 10.1016/j.wneu.2021.06.147
9. Bilir C, Cakir E, Gülbagci B, Altindis M, Toptan H, Guclu E, et al. COVID-19 Prevalence and Oncologic Outcomes of Asymptomatic Patients With Active Cancer Who Received Chemotherapy. *Acta Med Mediterr* (2021) 37(1):667–71. doi: 10.19193/0393-6384_2021_1_103
10. Saygideger Y, Sezan A, Candevir A, Saygıdeğer Demir B, Güzel E, Baydar O, et al. COVID-19 Patients' Sera Induce Epithelial Mesenchymal Transition in Cancer Cells. *Cancer Treat Res Commun* (2021) 28:100406. doi: 10.1016/j.ctarc.2021.100406
11. Palich R, Veyri M, Marot S, Vozy A, Gligorov J, Maingon P, et al. Weak Immunogenicity After a Single Dose of SARS-CoV-2 mRNA Vaccine in Treated Cancer Patients. *Ann Oncol* (2021) 32(8):1051–3. doi: 10.1016/j.annonc.2021.04.020
12. Turnquist C, Ryan BM, Horikawa I, Harris BT, Harris CC. Cytokine Storms in Cancer and COVID-19. *Cancer Cell* (2020) 38(5):598–601. doi: 10.1016/j.ccell.2020.09.019
13. Francescangeli F, De Angelis ML, Baiocchi M, Rossi R, Biffoni M, Zeuner A. COVID-19–Induced Modifications in the Tumor Microenvironment: Do They Affect Cancer Reawakening and Metastatic Relapse? *Front Oncol* (2020) 10:592891. doi: 10.3389/fonc.2020.592891
14. Francescangeli F, De Angelis ML, Zeuner A. COVID-19: A Potential Driver of Immune-Mediated Breast Cancer Recurrence? *Breast Cancer Res* (2020) 22 (1):20–2. doi: 10.1186/s13058-020-01360-0
15. Okoye JO, Ibekailo NS, Ngokere AA, Obioma O. Warthin Tumour-Associated Synchronous Neoplasia and COVID-19: Does SARS-CoV-2 Infection Increase the Risk of Benign Tumours and Cancer. *J Med Lab Sci* (2020) 30(4):11–25. doi: 10.5281/zenodo.4364910
16. Wei X, Su J, Yang K, Wei J, Wan H, Cao X, et al. Elevations of Serum Cancer Biomarkers Correlate With Severity of COVID-19. *J Med Virol* (2020) 92 (10):2036–41. doi: 10.1002/jmv.25957
17. Albiges L, Foulon S, Bayle A, Gachot B, Pommeret F, Willekens C, et al. Determinants of the Outcomes of Patients With Cancer Infected With SARS-CoV-2: Results From the Gustave Roussy Cohort. *Nat Cancer* (2020) 1 (10):965–75. doi: 10.1038/s43018-020-00120-5
18. Di Cosimo S, Tagliaferri B, Generali D, Giudici F, Agustoni F, Bernardo A, et al. Baseline Characteristics and Outcomes of Cancer Patients Infected With SARS-Cov-2 in the Lombardy Region, Italy (Aiom-L Corona): A Multicenter, Observational, Ambispective, Cohort Study. *Cancers* (2021) 13(6):1–15. doi: 10.3390/cancers13061324
19. Garg M. Epithelial-Mesenchymal Transition - Activating Transcription Factors - Multifunctional Regulators in Cancer. *World J Stem Cells* (2013) 5 (4):188–95. doi: 10.4252/wjsc.v5.i4.188
20. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The Epithelial-Mesenchymal Transition Generates Cells With Properties of Stem Cells. *Cell* (2008) 133(4):704–15. doi: 10.1016/j.cell.2008.03.027

21. Del Valle DM, Kim-Schulze S, Huang HH, Beckmann ND, Nirenberg S, Wang B, et al. An Inflammatory Cytokine Signature Predicts COVID-19 Severity and Survival. *Nat Med* (2020) 26(10):1636–43. doi: 10.1038/s41591-020-1051-9
22. Suarez-Carmona M, Lesage J, Cataldo D, Gilles C. EMT and Inflammation: Inseparable Actors of Cancer Progression. *Mol Oncol 2017/06/26.* (2017) 11 (7):805–23. doi: 10.1002/1878-0261.12095
23. Wang W, Nag S, Zhang R. Targeting the NF-κb Signaling Pathways for Breast Cancer Prevention and Therapy. *Curr Medicinal Chem* (2014) 22(2):264–89. doi: 10.2174/09298673216614110612431 5
24. Ma JH, Qin L, Li X. Role of STAT3 Signaling Pathway in Breast Cancer. *Cell Communicat Signaling* (2020) 18(1):1–13. doi: 10.1186/s12964-020-0527-z
25. Baghban R, Roshangar L, Jahanban-Esfahlan R, Seidi K, Ebrahimi-Kalan A, Jaymand M, et al. Tumor Microenvironment Complexity and Therapeutic Implications at a Glance. *Cell Communicat Signaling* (2020) 18(1):59. doi: 10.1186/s12964-020-0530-4
26. Wolczyk D, Zaremba-Czogalla M, Hryniewicz-Jankowska A, Tabola R, Grabowski K, Sikorski AF, et al. TNF-α Promotes Breast Cancer Cell Migration and Enhances the Concentration of Membrane-Associated Proteases in Lipid Rafts. *Cell Oncol (Dordrecht) 2016/04/04.* (2016) 39 (4):353–63. doi: 10.1007/s13402-016-0280-x
27. Hartman ZC, Poage GM, Den Hollander P, Tsimelzon A, Hill J, Panupinthu N, et al. Growth of Triple-Negative Breast Cancer Cells Relies Upon Coordinate Autocrine Expression of the Proinflammatory Cytokines IL-6 and IL-8. *Cancer Res* (2013) 73(11):3470–80. doi: 10.1158/0008-5472.CAN-12-4524-T
28. DeSantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A, et al. Breast Cancer Statistics, 2019. *CA* (2019) 69(6):438–51. doi: 10.3322/caac.21583
29. Comşa Ş, Cîmpean AM, Raica M. The Story of MCF-7 Breast Cancer Cell Line: 40 Years of Experience in Research. *Anticancer Res* (2015) 35(6):3147–54.
30. Mei J, Cai Y, Xu R, Yu X, Chen L, Ma T, et al. SARS-CoV-2 Receptor ACE2 Identifies Immuno-Hot Tumors in Breast Cancer. *bioRxiv* (2021). doi: 10.1101/2021.05.10.443377
31. Chavez KJ, Garimella SV, Lipkowitz S. Triple Negative Breast Cancer Cell Lines: One Tool in the Search for Better Treatment of Triple Negative Breast Cancer. *Breast Dis* (2010) 32(1–2):35–48. doi: 10.3233/BD-2010-0307
32. Hirano T, Murakami M. COVID-19: A New Virus, But a Familiar Receptor and Cytokine Release Syndrome. *Immunity* (2020) 52(5):731–3. doi: 10.1016/j.immuni.2020.04.003
33. Jung YJ, Isaacs JS, Lee S, Trepel J, Liu ZG, Neckers L. Hypoxia-Inducible Factor Induction by Tumour Necrosis Factor in Normoxic Cells Requires Receptor-Interacting Protein-Dependent Nuclear Factor κb Activation. *Biochem J* (2003) 370(3):1011–7. doi: 10.1042/BJ20021279
34. Ma Y, Ren Y, Dai ZJ, Wu CJ, Ji YH, Xu J. IL-6, IL-8 and TNF-α Levels Correlate With Disease Stage in Breast Cancer Patients. *Adv Clin Exp Med* (2017) 26(3):421–6. doi: 10.17219/acem/62120
35. Fernando RI, Castillo MD, Litzinger M, Hamilton DH, Palena C. IL-8 Signaling Plays a Critical Role in the Epithelial-Mesenchymal Transition of Human Carcinoma Cells. *Cancer Res* (2011) 71(15):5296–306. doi: 10.1158/0008-5472.CAN-11-0156
36. Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, et al. CD44+/CD24- Breast Cancer Cells Exhibit Enhanced Invasive Properties: An Early Step Necessary for Metastasis. *Breast Cancer Research : BCR* (2006) 8(5):R59–9. doi: 10.1186/bcr1610
37. Cai X, Cao C, Li J, Chen F, Zhang S, Liu B, et al. Inflammatory Factor TNF-α Promotes the Growth of Breast Cancer *via* the Positive Feedback Loop of TNFR1/NF-κb (and/or P38)/P-STAT3/HBXIP/Tnfr1. *Oncotarget* (2017) 8 (35):58338–52. doi: 10.18632/oncotarget.16873
38. Chung SS, Aroh C, Vadgama JV. Constitutive Activation of STAT3 Signaling Regulates hTERT and Promotes Stem Cell-Like Traits in Human Breast Cancer Cells. *PloS One* (2013) 8(12):e83971–1. doi: 10.1371/journal.pone.0083971
39. Das K, Paul S, Singh A, Ghosh A, Roy A, Ansari SA, et al. Triple-Negative Breast Cancer-Derived Microvesicles Transfer Microrna221 to the Recipient Cells and Thereby Promote Epithelial-to-Mesenchymal Transition. *J Biol Chem* (2019) 294(37):13681–96. doi: 10.1074/jbc.RA119.008619
40. Senigagliesi B, Samperi G, Cefarin N, Gneo L, Petrosino S, Apollonio M, et al. Triple Negative Breast Cancer-Derived Small Extracellular Vesicles as Modulator of Biomechanics in Target Cells. *bioRxiv* (2022). doi: 10.1101/2022.02.28.481921
41. Lucas JM, Heinlein C, Kim T, Hernandez SA, Malik MS, True LD, et al. The Androgen-Regulated Protease TMPRSS2 Activates a Proteolytic Cascade Involving Components of the Tumor Microenvironment and Promotes Prostate Cancer Metastasis. *Cancer Discov 2014/08/13.* (2014) 4(11):1310–25. doi: 10.1158/2159-8290.CD-13-1010
42. Li T, Wang L, Wang H, Li X, Zhang S, Xu Y, et al. Serum SARS-COV-2 Nucleocapsid Protein: A Sensitivity and Specificity Early Diagnostic Marker for SARS-COV-2 Infection. *Front Cell Infect Microbiol* (2020) 10:470. doi: 10.3389/fcimb.2020.00470
43. Shan D, Johnson JM, Fernandes SC, Suib H, Hwang S, Wuelfing D, et al. N-Protein Presents Early in Blood, Dried Blood and Saliva During Asymptomatic and Symptomatic SARS-CoV-2 Infection. *Nat Commun* (2021) 12(1):1931. doi: 10.1038/s41467-021-22072-9
44. Ogata AF, Maley AM, Wu C, Gilboa T, Norman M, Lazarovits R, et al. Ultra-Sensitive Serial Profiling of SARS-CoV-2 Antigens and Antibodies in Plasma to Understand Disease Progression in COVID-19 Patients With Severe Disease. *Clin Chem* (2020) 66(12):1562–72. doi: 10.1093/clinchem/hvaa213
45. Jana AK, Greenwood AB, Hansmann UHE. Presence of a SARS-COV-2 Protein Enhances Amyloid Formation of Serum Amyloid a. *bioRxiv* (2021). doi: 10.1101/2021.05.18.444723
46. Thalla DG, Jung P, Bischoff M, Lautenschläger F. Role of Extracellular Vimentin in Cancer-Cell Functionality and Its Influence on Cell Monolayer Permeability Changes Induced by SARS-CoV-2 Receptor Binding Domain. *Int J Mol Sci* (2021) 22(14):7469. doi: 10.3390/ijms22147469
47. Johnson BD, Zhu Z, Lequio M, Powers CGD, Bai Q, Xiao H, et al. SARS-CoV-2 Spike Protein Inhibits Growth of Prostate Cancer: A Potential Role of the COVID-19 Vaccine Killing Two Birds With One Stone. *Med Oncol* (2022) 39 (3):32. doi: 10.1007/s12032-021-01628-1
48. Qian Y, Lei T, Patel PS, Lee CH, Monaghan-Nichols P, Xin HB, et al. Direct Activation of Endothelial Cells by SARS-CoV-2 Nucleocapsid Protein Is Blocked by Simvastatin. *J Virol* (2021) 95(23):e0139621. doi: 10.1128/JVI.01396-21
49. Song S, Zhang Y, Ding T, Ji N, Zhao H. The Dual Role of Macropinocytosis in Cancers: Promoting Growth and Inducing Methuosis to Participate in Anticancer Therapies as Targets. *Front Oncol* (2021) 10:570108. doi: 10.3389/fonc.2020.570108

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Oncology

MINI REVIEW
published: 14 June 2022
doi: 10.3389/fonc.2022.924808

Epigenetic Mechanisms Influencing Therapeutic Response in Breast Cancer

Amaia Arruabarrena-Aristorena[1,2,3]* *and Eneda Toska*[4,5]*

[1] Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Bizkaia Technology Park, Derio, Spain, [2] Ikerbasque, Basque Foundation for Science, Bilbao, Spain, [3] Traslational Prostate Cancer Research Lab, CIC bioGUNE-Basurto, Biocruces Bizkaia Health Research Institute, Derio, Spain, [4] Department of Oncology, Sidney Kimmel Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [5] Department of Biochemistry and Molecular Biology, Johns Hopkins School of Public Health, Baltimore, MD, United States

The majority of breast cancers are estrogen receptor (ER)+ and agents targeting the ER signaling pathway have markedly increased survival for women with breast cancer for decades. However, therapeutic resistance eventually emerges, especially in the metastatic setting. In the past decade disrupted epigenetic regulatory processes have emerged as major contributors to carcinogenesis in many cancer types. Aberrations in chromatin modifiers and transcription factors have also been recognized as mediators of breast cancer development and therapeutic outcome, and new epigenetic-based therapies in combination with targeted therapies have been proposed. Here we will discuss recent progress in our understanding of the chromatin-based mechanisms of breast tumorigenesis, how these mechanisms affect therapeutic response to standard of care treatment, and discuss new strategies towards therapeutic intervention to overcome resistance.

Keywords: breast cancer, epigenetics, estrogen receptor - ESR1, endocrine therapy, transcription factor, chromatin regulation

OPEN ACCESS

Edited by:
Ariella Hanker, University of Texas Southwestern Medical Center, United States

Reviewed by:
Alberto Servetto, University of Naples Federico II, Italy

***Correspondence:**
Amaia Arruabarrena-Aristorena
aarruabarrena@cicbiogune.es
Eneda Toska
etoska1@jhmi.edu

Specialty section:
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

Received: *20 April 2022*
Accepted: *05 May 2022*
Published: *14 June 2022*

Citation:
Arruabarrena-Aristorena A and Toska E (2022) Epigenetic Mechanisms Influencing Therapeutic Response in Breast Cancer. Front. Oncol. 12:924808. doi: 10.3389/fonc.2022.924808

INTRODUCTION

Over 250,000 breast cancer cases are diagnosed in the US each year (1). The majority of breast cancers (70%) express estrogen receptor (ER) and are treated with agents targeting the ER signaling pathway (2). Endocrine therapy has markedly improved the lives of breast cancer patients for decades. More recently the addition of PI3K inhibitors (alpelisib) or CDK4/6 inhibitors (palbociclib, ribociclib, abemaciclib) to antiestrogens has significantly prolonged progression-free survival (PFS) in comparison to anti-estrogens alone in patients with ER+ metastatic breast cancer (3–6). However, *de novo* and acquired resistance to these treatments remains a major challenge and a high research and clinical priority (5, 7). Research over the past decade has unmasked a key contribution of disrupted chromatin and transcriptional regulatory processes to cancer and in particular, ER+ breast cancer. Laboratory-based functional genetic screens (siRNA, CRISPR), together with molecular profiling of biopsies from patients resistant to targeted therapies have unveiled chromatin modifiers and transcription factors linked to metastatic resistant tumors. Here we

review several key epigenetic mechanisms dictating breast cancer tumorigenesis and their roles in therapeutic response in ER+ breast cancer. We also discuss the role of epigenetic factors as promising new targets for overcoming therapeutic resistance in breast cancer.

EPIGENETIC MECHANISMS OF ER SIGNALING

ER is a member of the endocrine or steroid receptor subfamily of nuclear receptors, also known as Type I nuclear receptors. As members of this subfamily, ER and other nuclear receptors such as AR, share a ligand binding-driven activation mechanism, meaning they bind chromatin upon steroid stimulation. Moreover, they have a common structural domain distribution that ensures the presence of: a variable binding site for interaction with cooperating factors at the N-terminus, namely activation function 1 (AF1); a DNA binding domain (DBD); the interdomain hinge, which encompasses a nuclear localization sequence (Hinge); and a specific ligand-binding domain, which also enables the interaction with additional cofactors and is known as activation function 2 (LBD, AF2) (2). While these nuclear receptors exist at the plasma membrane (in their monomeric form) and in the nucleus (dimerized), the major pool of ER (85%) localizes to the nucleus upon estrogen stimulation. Upon the hormonal trigger ER monomers change conformation and dimerize (**Figure 1**). Dimerized ER is then translocated into the nucleus where it specifically binds at estrogen responsive elements (EREs), with the subsequent induction of the estrogen response (8). This leaves approximately 5% of ER, in its monomeric conformation, that travels to the cell surface upon palmitoylation and subsequently associates with Caveolin-1. ER relies on interactions with several co-regulator proteins to promote or inhibit its activity (**Figure 1**). Examples include the p160 family proteins (SRC1, GRIP1 and AIB1), namely co-activators, as well as nCOR1 and SMRT, which function as repressors Other cooperating factors consist of pioneer factors, such as FOXA1 (9) or GATA3 (10); ATP-dependent chromatin remodelers, for instance SWI/SNIF complex subunits BRG1, BRM or BAF57; and finally, histone and DNA modifiers, such as acetyltransferases (HATs), deacetylases (HDACs), methyltransferases and demethylases. p160 proteins are responsible for recruiting the co-activators p300 and CBP, and histone methyltransferases CARM1 and PRMT1. p300 and CBP have intrinsic and specific histone acetyltransferase (HAT) activity for H3K14, H4K5, H4K8 and additional lysine residues in histone 2A and 2B subunits. ER can also indirectly interact with other HATs. An example is p300/CBP-associated factor (PCAF), which can self-acetylate or be acetylated by p300, while it acetylates H3K9 and H3K14. On the other hand, PRMT1 is responsible for H4R3 methylation, while CARM1 methylates H3R2, H3R17 and H3R26. However, these methylation modifications are reversible through the action of the lysine-specific demethylase 1 (LSD1), which can specifically demethylate H3K4 and H3K9 (2). Bromodomain protein BRD4 has also been shown to be required for ER-dependent enhancer activation and transcription (11). Finally, ER also interacts with other epigenetic regulators, such as Polycomb repressive

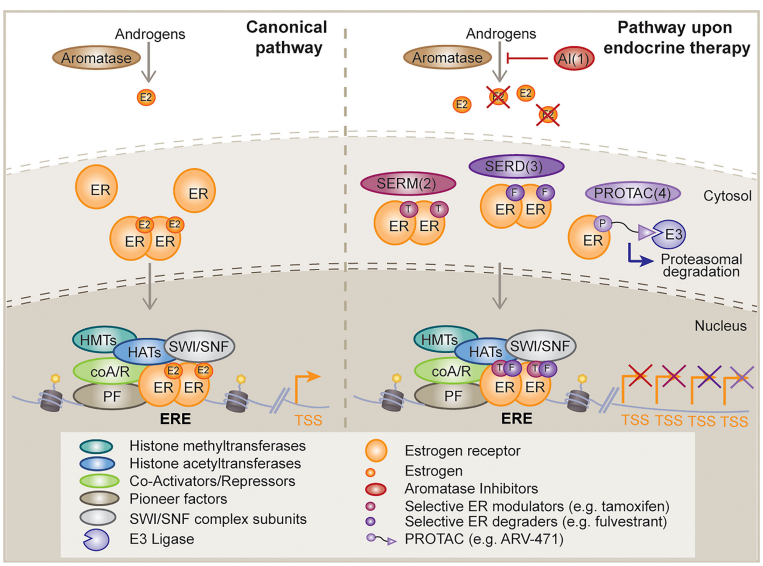


FIGURE 1 | Mechanism of action of classical and novel endocrine therapies. Schematic diagram depicting, on the left, the canonical estrogen receptor (ER) activating signaling cascade and potential downstream interactor types; and on the right, the different mechanisms of disruption of this signaling pathway upon distinct endocrine therapy strategies, such as, aromatase inhibitors (AI, 1), selective ER modulators (SERMs, 2), selective ER degraders (SERDs, 3) and proteolysis targeting chimeras (PROTACs, 4). E2, estrogen; ER, estrogen receptor; T, tamoxifen; F, fulvestrant; E3, E3 ligase; HMT, histone methyltransferase; HAT, histone acetyltransferase; coA/R, co-activator/repressor; PF, pioneer factor; TSS, transcription start site; ERE, estrogen response element.

complex 1 (PRC1) (12). RING1B, a core PRC1 subunit and a histone H2A ubiquitin ligase, is overexpressed in luminal breast cancers and is a crucial regulator of the dynamic, liganded-ER transcriptional programs (13).

GENOMIC ALTERATIONS AND STANDARD OF CARE TARGETED THERAPIES IN ER+ BREAST CANCER

Breast cancer was first demonstrated to be a hormone-driven disease by George Beatson in 1896 (14), long before the discovery of ER by Elwood Jensen and Jack Gorski in 1967 (15, 16). These findings ignited the development of endocrine therapies and personalized medicine. Currently, the ER signaling pathway is targeted by selective ER modulators (SERMs) (e.g., tamoxifen), which compete with estrogen for binding to ER; selective ER degraders (SERDs) (e.g., fulvestrant) that are thought to induce ER protein degradation or block ER activity; and aromatase inhibitors (AIs) (e.g., anastrozole, letrozole, exemestane), which deplete estrogen sources by inhibiting the conversion of androgens to estrogens (5) (**Figure 1**). Of note, it has been recently shown that a number of fulvestrant-like ER degraders suppress ER dependent-transcription mainly by slowing the intra-nuclear mobility of ER (17). In addition, a number of next generation oral SERDs with potentially better pharmacological properties than fulvestrant are in clinical trials (18) (**Figure 1**). These include rintodestrant (phase I, NCT03455270), elacestrant/RAD1901 (phase 3, NCT03778931), giredestrant/GDC-9545 (phase II, NCT04436744), amcenestrant/SAR439859 (phase III, NCT04478266), camizestrant/AZD-9833 (phase III, NCT04711252), and LY3484356 (phase I, NCT04188548) among others. Recent press news has revealed that giredestrant and amcenestrant did not meet their primary endpoint of improving progression free survival (PFS) while the EMERALD trials of elecastrant showed a 30% reduction in PFS during the 2021 San Antonio Breast Cancer Symposium. Novel therapies that are also in the clinic include the SERMs lasofoxifene (phase II, NCT03781063), bazedoxifene (phase I/2, NCT02448771), the proteolysis-targeting chimeras (PROTAC) ARV-471 (phase 1/2, NCT04072952), and the selective estrogen receptor covalent antagonist (SERCA) H3B-5942 (phase I, NCT04288089). Preclinical work has shown significant single-agent antitumor activity of H3B-5942 in wild-type ER and mutant ER xenograft models that was superior to fulvestrant and whose potency could be improved further in combination with CDK4/6 or mTOR inhibitors (19). The development of these new bioavailable drugs against ER raises hopes that they may improve the lives of patients with resistant ER+ breast cancer.

One of the hallmarks of ER+ breast cancer is its dependence on the phosphatidylinositol-3-kinase (PI3K) pathway, which is highlighted by the frequency of activating mutations in the gene *PIK3CA* (~40%), coding for the catalytic subunit of PI3K. Other alterations that can lead to hyperactivation of the PI3K pathway in breast cancer include *ERBB2* and *AKT* mutations, and deletions, nonsense and loss-of-function missense mutations in the tumor suppressor PTEN (20, 21). Aberrant activation of the PI3K pathway promotes acquired resistance to anti-ER therapies in preclinical models (22, 23). The clinical significance of the PI3K pathway in ER+ breast cancer has been shown by the approval of PI3K pathway inhibitors in this setting. The mTORC1 inhibitor everolimus, which inhibits a critical PI3K pathway node was approved first in combination with AIs in metastatic breast cancer patients that are refractory to endocrine therapy (24, 25). More recently, in patients with metastatic ER +/*PIK3CA* mutant breast cancer, the addition of the PI3Kα inhibitor alpelisib was approved in combination with fulvestrant (4). The AKT inhibitor capivasertib in combination with fulvestrant has also shown benefit in preliminary studies in endocrine refractory ER+ breast cancer. This combination may be effective in *AKT* or *PTEN* mutant breast cancer (26).More recently, it has been shown that proline rich 11 (PRR11) overexpression amplifies PI3K signaling and promotes endocrine therapy resistance in breast cancer, suggesting that the ER+/PRR11-amplified breast cancers subgroup of tumors can also benefit from treatment with PI3K inhibitors and antiestrogens (27).

While ER and PI3K pathway alterations are the most frequent oncogenic drivers in ER+ breast cancers, other drivers such as cyclin D1 are expressed at a high level, with or without gene amplification. ER activates the CCND1 promoter, while cyclin D1 also binds to and facilitates ER transcriptional activity, reflecting the possible dependence of ER+ tumors on cyclin D1 to initiate the G1-to S-phase transition. Accordingly, addition of CDK4/6 inhibitors (e.g., palbociclib, ribociclib, abemaciclib) to anti-ER therapy have markedly prolonged survival compared to anti-ER therapy alone in ER+ metastatic breast cancers (3, 6). Thus, after decades of endocrine therapy as a single agent the approval of everolimus, alpelisib, and CDK4/6 inhibitors has led to significant progress in breast cancer management. ERBB2 amplification/HER2 overexpression is also found in 10% of ER+ breast cancers and the current standard of care for ER+/HER2+ is a combination of anti-ER and HER2 inhibitors (28). Rare HER2 mutants found in 5% of endocrine-resistance metastatic breast cancer have also been associated with endocrine resistance (29). However, the combination of the HER2 inhibitor neratinib with fulvestrant has shown promise in this setting (30).

An enrichment in mutations in genes coding for transcription factors (TFs), such as GATA3, CTCF, FOXA1, and MYC (31); and chromatin modifiers, such as the histone methyltransferases (KMT2B, KMT2D, KMT2E) and histone demethylases (KDM4A, KDM5B, KDM5C, KDM6A) (20), and SWI/SNF complex subunits (ARID1A, ARID2) (31), have also been observed in ER+ breast cancer. However, the functional relevance of most of these alterations remain to be identified. More recently, the Breast International Group (BIG) molecular screening initiative AURORA identified a driving role for somatic mutations in the TF *GATA1* and the chromatin regulator *MEN1* among 381 breast cancer patients (32). Apart

from *TP53*, *PIK3CA*, *ESR1*, and *GATA3*, the most frequent alterations in primary and/or metastases in the AURORA cohort were found in the lysine histone acetyltransferase KAT6A (32). KAT6A is also amplified as part of the 8p11 amplicon in 10-15% of breast cancers. In addition to the aforementioned alterations, breast cancers also harbor a variety of rare mutations with low prevalence across subtypes, highlighting the heterogeneity of breast cancer and the need to study these variants to develop targeted therapies matched to the specific molecular alteration of each patient's tumor.

TRANSCRIPTION FACTORS AND CHROMATIN MODIFIERS AFFECTING THERAPEUTIC OUTCOME IN ER+ BREAST CANCER

In the contemporary era, next-generation sequencing technologies, such as whole genome sequencing (WGS) and whole-exome sequencing (WES), have expanded the landscape of genomic variations occurring in cancer, particularly in hormone-dependent breast cancer. Among the most frequently altered genes we find a variety of transcription factors and chromatin remodelers (31, 33). We will focus this part of the review on those examples proven to directly or indirectly impact patient response to standard of care treatment. For more detailed review of chromatin-based mechanisms in breast cancer see Morey and colleagues (7).

Alterations in Transcription Factors Affecting Endocrine Therapy Response

Despite the initial success of targeted endocrine therapies to tackle ER-driven programs, resistance to such treatments eventually emerges. Mutations in ER itself are a prominent example of driver alterations. Recurrent *ESR1* mutations localized at the ligand-binding domain (e.g. mutations at residues T537 and D538) have been shown to confer ligand-independent activity, establishing a range of sensitivity to the distinct ER antagonists and hormone depleting agents, such as AIs (34–36). Besides promoting a constitutively active agonist conformation, these alterations lead to an altered ER cistrome and the induction of a pro-metastatic transcriptome (37). ER relies on multiple cooperating factors, such as pioneer factors and coregulators, to regulate the estrogen response. FOXA1 is a driver of luminal breast cancer identity (38), and a crucial pioneer and cooperating factor for nuclear receptor activity (9, 39, 40). Recent work aimed at elucidating the mechanisms that regulate FOXA1 binding to the chromatin, has identified the lysine-specific demethylase 1A (LSD1) to positively regulate FOXA1 binding by demethylating lysine 270 on FOXA1 (41). LSD1 inhibition affected androgen response in prostate cancer and sensitized tumors further to anti-AR therapy (41). We have also shown that FOXA1 binding profiles are influenced by the SWI/SNF complex (42) and the histone methyltransferase KMT2D in breast cancer (43), suggesting a possible role for the chromatin landscape to evoke further differences in DNA binding for FOXA1.

Our work and others have also shown that genomic disturbances in *FOXA1* can alter ER transcriptional dynamics, driving endocrine therapy resistance. Specifically, activating missense mutations in the Wing2 loop (e.g. H247Y, S250F, F266L) increase the recruitment of FOXA1 to ER cis-regulatory elements and enhance ER-mediated transcription. Breast cancer-specific mutation SY242CS, on the other hand, incites chromatin accessibility changes, leading to the induction of alternative transcriptomes. Moreover, these and other hotspot mutations were found to be mutually exclusive with *ESR1* mutations, and associated with a poorer response to AI therapy in patients (44). In the same line, gene amplification or mutations at the *FOXA1* promoter induce enhanced FOXA1 expression and resistance to standard of care ER degraders and modulators respectively (45, 46) (**Figure 2**). While recent studies characterized the functional outcome of distinct *FOXA1* alterations *in vitro* in other hormone-related cancers, such as prostate cancer (PCa), further *in vivo* studies are required to assess their effect in response to androgen deprivation therapy (47, 48).

Alterations in a variety of ER-related pioneer factors have also been associated with endocrine therapy resistance. One example is GRHL2, a transcription factor classically known for its involvement in epithelial morphogenesis and differentiation, that has been recently characterized as a pioneer factor (49). This novel chromatin opener is enriched at ER loci and cooperates with FOXA1 to drive endocrine therapy resistance in luminal breast cancer. Moreover, increased GRHL2 protein levels are associated with reduced responsiveness to tamoxifen treatment (50, 51). This transcription factor is also amplified in prostate cancer, where it colocalizes with and regulates AR. Its role in the therapeutic response in this context however is ill-defined (52). Another important example is PBX1, which co-occupies 85% of ER loci. Magnani et al. showed that, in fact, this transcription factor is necessary to induce an estrogen-dependent transcriptome distinct from that activated by FOXA1. In line with this, FAIRE-seq experiments revealed that PBX1-bound chromatin is rendered accessible in the absence of estrogen stimulation, demonstrating its pioneering capacity (53). PBX1 has also been shown to regulate ER-dependent transcription upon PI3K inhibition and to sensitize breast cancer cells further to alpelisib (43). Moreover, PBX1 is known to be amplified in metastatic ER+ breast cancers. Importantly, disease-free survival analysis of luminal breast cancer patients from TCGA uncovered PBX1 amplification as a potential biomarker with prognostic value, while the family member with prognostic capacity in PCa has been suggested to be PBX3 (54, 55). PBX1 mediates the expression of a unique NOTCH3 transcriptome that drives endocrine therapy resistance and reduces metastasis-free survival in ER+ cancers (56).

Another bona fide pioneer factor for ER is GATA3, which is mutated in 17% of ER+ metastatic breast cancers (5). However,

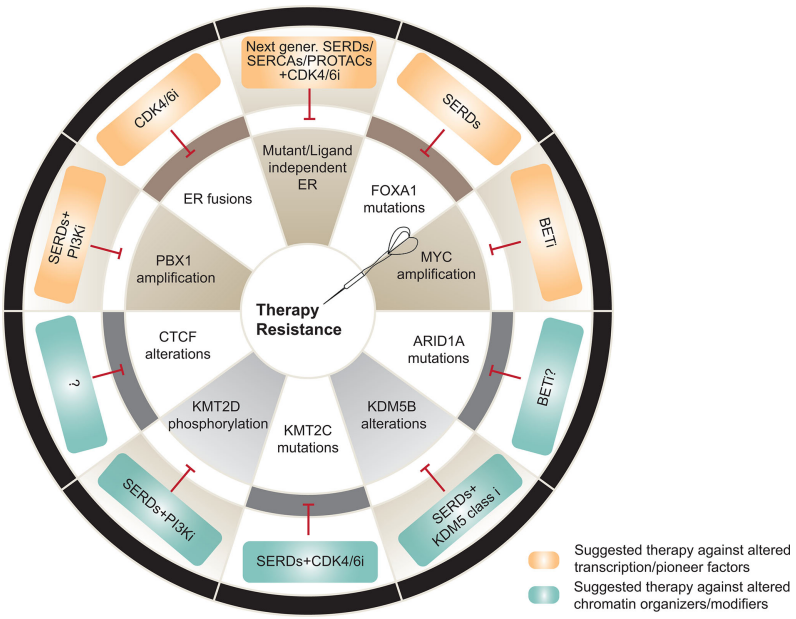


FIGURE 2 | Transcriptional and epigenetic mechanisms of therapy resistance and potential therapeutic alternatives to overcome them. Top, Alterations in transcription and pioneer factors involved in resistance (brown) and suggested therapeutic strategies against the driven mechanisms (orange). Bottom, Alterations in chromatin organizers and modifiers associated to resistance (grey) and suggested therapeutic strategies against the driven mechanisms (blue). SERD, selective estrogen receptor degraders; SERCA, selective ER covalent antagonists; PROTAC, proteolysis-targeting chimeras. Figure adapted and modified from (5).

whether these genomic alterations predict better or worse prognosis in breast cancer remains a controversial issue. In fact, while some studies suggest that *GATA3* mutant tumors might have better overall survival (OS) (57), other groups found no difference in OS, but observed better prognosis for *GATA3* WT tumors (58). Moreover, a comprehensive massively parallel sequencing analysis of 77 tumors suggested that *GATA3* mutations could be positive predictive markers for aromatase inhibitor response (59). The limited experimental evidence suggests that frameshift alterations in this gene might provide a growth advantage compared to cells harboring the WT version. However, these experiments were only conducted in the context of estrogen supplementation, and had no effect on sensitivity to a panel of endocrine treatments or chemotherapies. Furthermore, the mutational repertoire represented in this study only covered a specific frameshift mutation in the ZF2 domain, while leaving most of the truncating alterations in *GATA3* unexplored (31, 60). Evidence suggests that GATA2 is the family member with an equivalent role in regulating the AR cistrome in prostate cancer (61). In addition, GATA2 expression is upregulated upon chemotherapy, driving CRPC aggressiveness (62).

ER activity is also regulated by the pioneer function of AP-2. The gene encoding for this transcription factor, TFAP2, is amplified in 4% of luminal breast cancers and its gene and protein expression levels are associated with worse progression free survival (PFS) upon fulvestrant treatment (63). Magnani and colleagues identified another TF, namely YY1 to be associated with clonal enhancers and promoters in breast cancer patients and as a novel critical determinant of ER transcriptional activity (64).

The pro-oncogene MYC is frequently amplified and is a driver of aberrant proliferation and aggressiveness in many tumor types, including basal breast cancers (65). Along with its well-characterized role in triple-negative or basal-like cancers MYC has also been associated with endocrine therapy resistance mechanisms (66, 67). Another important regulator of the ER transcriptional program is the DNA-binding protein CTCF, which is also found to be mutated in luminal breast cancers (20, 68). Recently, single-nucleotide variation (SNVs) at CTCF binding sites have been associated with altered interaction patterns and transcription of ER target genes, leading to endocrine therapy resistance (69). Resistance-associated SNVs were also strongly enriched at ER binding sites. ER reprogramming in endocrine resistant cells was associated with rewiring of ER-bound interactions between active enhancers and promoters and aberrant expression of these target genes, with many of them being involved in ER-signaling and therapy outcome (69). This work suggested that 3D epigenome remodeling may be an important mechanism underlying endocrine therapy resistance in ER+ breast cancer.

Treatment with PI3K (43) or CDK4/6 inhibitors (70) have also been shown to remodel the chromatin landscape of breast cancer, specifically at enhancers. In regards to CDK4/6 inhibitors, AP-1 transcription factors were upregulated on treatment, which in turn were implicated with widespread enhancer activation in breast tumor models (70). PI3K

inhibitors on the other hand enhanced chromatin accessibility at ER cis-regulatory elements (discussed at section 6) (43). Further work is needed to delineate the chromatin landscape of breast tumors resistant to PI3K or CDK4/6 inhibitors.

Finally, several coregulators involved in the activation or repression of the ER machinery have been reported to be recurrently altered in metastatic breast cancers. Examples are NCOR1 (71), RUNX, RARA and AP1. However, functional evidence has yet to be gathered in order to establish them as drivers of endocrine therapy resistance. The case is similar for other transcription factors reported to be mutated in metastatic breast cancers, such as TBX3 or CBFB (31, 33).

For a recent comprehensive review on endocrine therapy resistance mechanisms see Hanker and colleagues (5).

Alterations in Chromatin Remodelers Impacting Endocrine Therapy Outcome

Truncating mutations in *ARID1A* imply loss of function of this SWI/SNF chromatin remodeling complex subunit in ER+ breast cancers (31, 32). Loss of function mutations or deletions in the SWI/SNF nucleosome remodeling components ARID1A and ARID2 are also enriched in metastatic endocrine-resistant breast cancer (31). We and others recently reported that ARID1A loss is associated with a shorter response to SERDs (11, 42). Mechanistically, ARID1A loss reduces chromatin accessibility and SWI/SNF complex binding at the loci of luminal-determining TFs like FOXA1, ER, and GATA3, resulting in a downregulation of luminal gene signatures and a subset of estrogen regulated genes. These findings may provide an explanation for the longstanding clinical observation that ER+ breast tumors exposed to therapy eventually lose ER and become endocrine therapy resistant. Therapeutic pressure may enable the emergence of cells harboring loss of function mutations in *ARID1A* that confer independence from ER (**Figure 2**). Enhancer reprogramming which promotes phenotypic plasticity and endocrine therapy resistance in breast cancer has also been observed to be mediated by the coordinated role of GATA3 and AP1 TFs which re-organize enhancer landscape promoting tumor phenotypic plasticity (72). Prostate cancer also utilizes similar mechanisms to overcome androgen- and AR target therapies. It has been shown that lineage plasticity can also promote anti-androgen resistance through the SOX2 transcription factor in a TP53-and RB1 loss background in prostate cancer (73). We anticipate that additional alterations in epigenetic and transcriptional regulators are responsible for lineage plasticity upon therapy in hormone-driven cancers.

Sensitivity to endocrine therapies is impacted in a similar fashion by the perturbed action of chromatin modifiers, such as histone methyltransferases or demethylases. For instance, loss of KMT2C (namely MLL3), one of the six members of the SET family of histone lysine methyltransferases, is reported to drive hormone independence in ER+ breast cancer. KMT2C is one of most mutated or deleted genes in ER+ breast cancer patients, and is associated with shorter disease-free survival upon estrogen deprivation with AIs. Despite the advantage of KMT2C-depleted cells in estrogen-deprived conditions, these cells remain ER-dependent and thus, sensitive to therapies involving ER degraders or modulators (74). Another member of the family, KMT2D, happens to be frequently mutated in this cancer type. While there is not enough scientific evidence to relate these alterations to endocrine therapy sensitivity, our group demonstrated that loss of KMT2D sensitizes breast cancer further to PI3K inhibitors through the downregulation of the ER signaling cascade (43). Thus, it is tempting to hypothesize that loss of function mutations in KMT2D might increase sensitivity to ER-targeted therapies. On the other hand, H3K4 demethylases, such as KDM5 (or JARID1B), have been established as oncogenes in luminal ER breast cancer due to their frequent amplification or overexpression (75). In fact, high levels of KDM5 are reported to increase transcriptional heterogeneity, leading to selection of pre-existing resistant clones and poor prognosis in ER+ breast cancers (76).

CROSS-TALK BETWEEN SIGNALING PATHWAYS AND HORMONE RECEPTORS

One of the first evidences of PI3K and ER signaling crosstalk came from the Breast Cancer Trials of Oral Everolimus-2 (BOLERO-2) phase III clinical trial which demonstrated improvement in progression-free survival (PFS) in endocrine resistant ER+ breast cancer patients treated with the mTOR inhibitor everolimus and exemestane (24). As the first PI3K inhibitors were emerging in the clinic, we and others studied their effects on ER signaling with the goal of identifying the most effective combinatorial therapy for ER+/*PIK3CA* mutant breast cancer. In this regard, we observed a highly uniform adaptive mechanism, orchestrated by the activation of ER signaling upon PI3Kα inhibition, that limited sensitivity to PI3K inhibitors and could be reversed by the addition of endocrine therapy (77). These preclinical findings paved the way for phase III clinical studies testing the PI3Kα inhibitor alpelisib with fulvestrant in patients with metastatic *PIK3CA*-mutant ER+ breast cancer and culminated in the approval of alpelisib by the Food and Drug Administration (FDA) in 2019 (4). Of note, in prostate cancer, which is also dependent on AR and PI3K signaling, it has been shown that inhibition of the PI3K pathway activates AR signaling to support tumor survival. Thus, inhibition of oncogenic PI3K increases tumor growth by unleashing ER/AR signaling in breast and prostate respectively. Mechanistically, PI3Kα inhibition enhances ER signaling through loss of phosphorylation of the epigenetic regulator KMT2D by the PI3K effectors AKT and SGK (43, 78), providing a rationale for epigenetic therapy in combination with PI3K inhibition in this setting. For recent reviews on PI3K inhibitors for cancer therapy see (79–81).

HER2 overexpression has been shown to mediate resistance to endocrine therapies through activation of PI3K or MAPK signaling pathways and thus, ER+ HER2+ patients are currently treated with endocrine therapy in combination with HER2 inhibitors (5). More recently, HER2 activating mutations were found in ~5% of endocrine-resistant metastatic breast cancer

(31) and were shown to play active roles in driving resistance (29). Combining anti-HER2 therapy neratinib with fulvestrant has proved to be an effective therapeutic strategy for these tumors (30). In addition, alterations in the MAPK pathway such as NF1 loss are frequent in endocrine resistant ER+ metastatic breast cancer (31), and contribute to resistance to fulvestrant *via* both ER-dependent and ER-independent mechanisms (82). Moreover, nuclear RTKs like FGFR1 have also been shown to influence gene expression in ER+ breast cancer and mediate endocrine therapy resistance (83).

COLLABORATIVE CROSSTALK OF NUCLEAR HORMONE RECEPTORS

Nuclear receptors events in breast cancer have been generally studied as single receptor chromatin binding events. However, it has become apparent that nuclear receptors collaborate with each other to influence each other binding and therapeutic response with greater complexity than previously recognized. Carroll and colleagues (84) have reported that activated progesterone receptor (PR) can reprogram ER enhancer landscape and that progesterone inhibits estrogen-mediated growth of ER increasing the anti-proliferative effects of endocrine therapy. AR has also been shown to facilitate ER chromatin binding and AR inhibition reduced estradiol-mediated proliferation in ER+/AR+ breast cancer cell lines and synergized with tamoxifen and fulvestrant (85). The role of glucocorticoid receptor (GR) and its post-translational modification of GR such as SUMOylation has been also shown to induce or repress a number of ER binding events and potentially influence decisions on breast cancer therapies (86). However, it is still unclear the chromatin-based mechanisms associated with these crosstalk among nuclear receptors.

NEW AVENUES OF EPIGENETIC THERAPY

Precision oncology efforts have led to the development of epigenetic drugs and nine drugs are FDA-approved including inhibition of EZH2, IDH, DNMTs, and HDACs. Multiple others are in clinical trials for both solids and hematological malignancies. In ER+ breast cancer, phase II trials (NCT00676663, NCT04190056, NCT00828854) are testing therapeutic efficacy of epigenetic drugs with standard of care therapies. Recently, the HDAC inhibitor entinostat has been explored to re-sensitize ER+ tumors to endocrine therapy (ENCORE301) (NCT00676663) but unfortunately has failed to overcome resistance (results presented by M Connolly et al, San Antonio Breast Cancer Symposium, 2020). HDAC inhibitors are also in clinical trials in combinations with CDK4/CDK6 inhibitors (ribociclib, NCT04315233) in triple negative breast cancer. Histone acetylation catalyzed by histone acetyltransferases such as p300/CBP have been shown to be increased in endocrine resistant breast cancer cells highlighting a need to better understand the role of protein acetylation in breast cancer (7). Interestingly, selective inhibitors against p300/CBP, namely CCS1477 has been shown to inhibit the AR transcription program and is currently being evaluated in clinical trials for metastatic castration resistance prostate cancer (NCT03568656). A better understanding of the epigenetic mechanisms influencing breast cancer progression and therapeutic response will be needed for novel drug discovery efforts and rationale-combinatory treatments.

We have learned thus far, that epigenetic regulators have been implicated in endocrine therapy resistant tumors where they can affect ER-dependent transcription, alter the network of ER cofactors or its crosstalk with other signaling pathways, or induce lineage differentiation to promote tumorigenesis. For instance, tumors with high KDM5B have been associated with a shorter response to endocrine treatment, suggesting that inhibitors of the KDM5 family could improve the response to endocrine agents. Likewise, loss of function mutations in KMT2C or mutations in *FOXA1* have been associated with a shorter response to AIs, making ER degraders such as fulvestrant the optimal therapeutic option for the tumors harboring these alterations. In the case of *ESR1* LBD mutations, decreased response to AIs is accompanied of a reduced sensitivity to fulvestrant (35) requiring alternative strategies, such as next generation SERDs, (SERCAs) or PROTACs (19, 87–90).

Other studies have shown how loss of function mutations in *ARID1A* are associated with SERD resistance (42, 91). One of the therapeutic strategies explored in *ARID1A* mutant cancers has been synthetic lethality. To this end, Carroll and colleagues have suggested exploiting synthetic lethality-based treatment strategies in *ARID1A* mutant cancers using inhibitors of BET proteins (91). Similar strategies have been proposed for *ARID1A* mutant ovarian cancer targeting the methyltransferase EZH2 (92). Epigenetic regulators such as KMT2D have also been shown to sensitize ER-driven tumors further to PI3K inhibitors suggesting that small molecule inhibitors against KMT2D could be a promising therapeutic choice in combination with PI3K inhibitors and endocrine therapy (43). Indeed, the development of small molecules that target chromatin regulators has emerged as an active area of current drug discovery efforts. For instance, given the *KAT6A* amplification in 10-15% of breast cancers, novel compounds against KAT6A/KAT68 (PF-9363) have been developed and analyses in preclinical models demonstrate potent anti-tumor activity in ER+ breast cancer cells and xenografts with KAT6A dysregulation (93).

CONCLUSION

Over the past decade, the field of transcription and chromatin regulation has grown tremendously and new chromatin-associated processes have emerged as drivers of tumor development and therapeutic response in hormone-driven cancers. These findings have been potentiated by the genomic, transcriptomic, whole-exome, and chromatin accessibility sequencing of breast tumors and preclinical mechanism-based studies using CRISPR-Cas9 screens and whole-genome epigenomic sequencing such as HI-C,

CUT & RUN, ATAC-seq and others. Specifically, genomic and transcriptomic analyses of primary breast cancer tumors and matched metastases, coupled with highly curated clinical data, from MSK-IMPACT or AURORA (BIG) initiatives have identified alterations in epigenetic regulators enriched in relapsed metastatic breast cancer (32, 94, 95). A number of these chromatin regulatory processes have begun to be validated and mechanistically delineated in the lab. The systematic integration of such multi-omics analyses of paired biopsies in clinical practice coupled with preclinical mechanistic validation will allow the identification of uncharacterized epigenetic drivers of breast tumorigenesis and therapeutic outcome. In addition, the rapid adoption of technologies that detect circulating tumor-derived cfDNA along with single-cell RNA/ATAC-sequencing will be important to capture the molecular heterogeneity of treatment resistance. Given that some of the genomic mechanisms of endocrine resistance have been found to be at low frequency, future efforts will require greater patient sample size and a focus not only on genomics on a panel of genes but whole-exome sequencing and transcriptomics and chromatin accessibility analyses to provide signatures of therapeutic resistance and response. These efforts would be facilitated by multi-institutional and cooperative data sharing efforts similar to the AURORA initiative. Finally, the identification of novel epigenetic regulators as drivers of breast tumorigenesis and therapeutic response will allow the rational design of novel inhibitors to overcome resistance. In order for these mechanisms to be suitable targets for cancer therapy, future work will need to identify: i) the tumor subtypes that are highly addicted to the chromatin-based mechanism, ii) rationale-based combinatorial strategies, and iii) optimal dosing and scheduling to increase efficacy and safety. This new and exciting body of evidence together with the systematic and integrative pursuit of multi-omics approaches in preclinical and clinical samples will greatly impact the study of chromatin regulatory systems in breast cancer and the identification of new treatment strategies.

AUTHOR CONTRIBUTIONS

AA-A and ET jointly participated in the planning, writing, and editing of the review. All authors contributed to the article and approved the submitted version.

FUNDING

AA-A is supported by a Juan de la Cierva Incorporacion fellowship from the Ministerio de Ciencia e Innovacion (Spain) and by a European Research Council Consolidator grant. ET is supported by grants from the JKTG foundation, Breast Cancer Research Alliance, Cancer Research Informatics, and NCI NIH grants K22CA245487 and R21CA252530.

ACKNOWLEDGMENTS

We would like to thank Dr. Ryan Blawski for edits on the manuscript.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA: A Cancer J Clin* (2017) 67:7–30. doi: 10.3322/CAAC.21387
2. Green KA, Carroll JS. Oestrogen-Receptor-Mediated Transcription and the Influence of Co-Factors and Chromatin State. *Nat Rev Cancer* (2007) 7:713–22. doi: 10.1038/nrc2211
3. Turner NC, Ro J, André F, Loi S, Verma S, Iwata H, et al. Palbociclib in Hormone-Receptor–Positive Advanced Breast Cancer. *New Engl J Med* (2015) 373:209–19. doi: 10.1056/NEJMOA1505270
4. André F, Ciruelos E, Rubovszky G, Campone M, Loibl S, Rugo HS, et al. Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer. *New Engl J Med* (2019) 380:1929–40. doi: 10.1056/NEJMOA1813904
5. Hanker AB, Sudhan DR, Arteaga CL. Overcoming Endocrine Resistance in Breast Cancer. *Cancer Cell* (2020) 37:496–513. doi: 10.1016/j.ccell.2020.03.009
6. Spring LM, Wander SA, Andre F, Moy B, Turner NC, Bardia A. Cyclin-Dependent Kinase 4 and 6 Inhibitors for Hormone Receptor-Positive Breast Cancer: Past, Present, and Future. *Lancet (London England)* (2020) 395:817–27. doi: 10.1016/S0140-6736(20)30165-3
7. Garcia-Martinez L, Zhang Y, Nakata Y, Chan HL, Morey L. Epigenetic Mechanisms in Breast Cancer Therapy and Resistance. *Nat Commun* (2021) 12:1–14. doi: 10.1038/s41467-021-22024-3
8. Kumar V, Chambon P. The Estrogen Receptor Binds Tightly to its Responsive Element as a Ligand-Induced Homodimer. *Cell* (1988) 55:145–56. doi: 10.1016/0092-8674(88)90017-7
9. Caroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, et al. Chromosome-Wide Mapping of Estrogen Receptor Binding Reveals Long-Range Regulation Requiring the Forkhead Protein Foxa1. *Cell* (2005) 122:33–43. doi: 10.1016/J.CELL.2005.05.008
10. Theodorou V, Stark R, Menon S, Carroll JS. GATA3 Acts Upstream of FOXA1 in Mediating ESR1 Binding by Shaping Enhancer Accessibility. *Genome Res* (2013) 23:12. doi: 10.1101/GR.139469.112
11. Nagarajan S, Hossan T, Alawi M, Najafova Z, Indenbirken D, Bedi U, et al. Bromodomain Protein BRD4 is Required for Estrogen Receptor-Dependent Enhancer Activation and Gene Transcription. *Cell Rep* (2014) 8:460–9. doi: 10.1016/J.CELREP.2014.06.016
12. Chan HL, Beckedorff F, Zhang Y, Garcia-Huidobro J, Jiang H, Colaprico A, et al. Polycomb Complexes Associate With Enhancers and Promote Oncogenic Transcriptional Programs in Cancer Through Multiple Mechanisms. *Nat Commun* (2018) 9:1–16. doi: 10.1038/s41467-018-05728-x
13. Zhang Y, Zhang Y, Chan HL, Chan HL, Garcia-Martinez L, Garcia-Martinez L, et al. Estrogen Induces Dynamic Erα and RING1B Recruitment to Control Gene and Enhancer Activities in Luminal Breast Cancer. *Sci Adv* (2020) 6:7249–54. doi: 10.1126/SCIADV.AAZ7249/SUPPL_FILE/AAZ7249_SM.PDF
14. Beatson GT. Meeting IX.—May 20, 1896: On the Treatment of Inoperable Cases of Carcinoma of the Mamma: Suggestions for a New Method of Treatment, With Illustrative Cases. *Trans Medico-Chirurgical Soc Edinburgh* (1896) 15:153. doi: 10.1016/s0140-6736(01)72307-0
15. Toft D, Shyamala G, Gorski J. A Receptor Molecule for Estrogens: Studies Using a Cell-Free System. *Proc Natl Acad Sci United States America* (1967) 57:1740. doi: 10.1073/PNAS.57.6.1740
16. Jensen EV, Suzuki T, Kawashima T, Stumpf WE, Jungblut PW, DeSombre ER. A Two-Step Mechanism for the Interaction of Estradiol With Rat Uterus. *Proc Natl Acad Sci United States America* (1968) 59:632–8. doi: 10.1073/PNAS.59.2.632
17. Guan J, Zhou W, Hafner M, Blake RA, Chalouni C, Chen IP, et al. Therapeutic Ligands Antagonize Estrogen Receptor Function by Impairing Its Mobility. *Cell* (2019) 178:949–963.e18. doi: 10.1016/J.CELL.2019.06.026
18. Fanning SW, Greene GL. Next-Generation Erα Inhibitors for Endocrine-Resistant ER+ Breast Cancer. *Endocrinology* (2019) 160:759–69. doi: 10.1210/EN.2018-01095
19. Puyang X, Furman C, Zheng GZ, Wu ZJ, Banka D, Aithal K, et al. Discovery of Selective Estrogen Receptor Covalent Antagonists for the Treatment of

Erα≪Sup<WT</sup< and Erα≪Sup<MUT</sup< Breast Cancer. *Cancer Discovery* (2018) 8:1176–1193. doi: 10.1158/2159-8290.CD-17-1229
20. TCGA. Comprehensive Molecular Portraits of Human Breast Tumours. *Nature* (2012) 490:61–70. doi: 10.1038/NATURE11412
21. Ciriello G, Gatza ML, Beck AH, Wilkerson MD, Rhie SK, Pastore A, et al. Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. *Cell* (2015) 163:506–19. doi: 10.1016/J.CELL.2015.09.033
22. Miller TW, Hennessy BT, González-Angulo AM, Fox EM, Mills GB, Chen H, et al. Hyperactivation of Phosphatidylinositol-3 Kinase Promotes Escape From Hormone Dependence in Estrogen Receptor-Positive Human Breast Cancer. *J Clin Invest* (2010) 120:2406–13. doi: 10.1172/JCI41680
23. Sanchez CG, Ma CX, Crowder RJ, Guintoli T, Phommaly C, Gao F, et al. Preclinical Modeling of Combined Phosphatidylinositol-3-Kinase Inhibition With Endocrine Therapy for Estrogen Receptor-Positive Breast Cancer. *Breast Cancer Res* (2011) 13. doi: 10.1186/BCR2833
24. Baselga J, Campone M, Piccart M, Burris HA, Rugo HS, Sahmoud T, et al. Everolimus in Postmenopausal Hormone-Receptor-Positive Advanced Breast Cancer. *New Engl J Med* (2012) 366:520–9. doi: 10.1056/NEJMOA1109653
25. Hortobagyi GN, Chen D, Piccart M, Rugo HS, Burris HA, Pritchard KI, et al. Correlative Analysis of Genetic Alterations and Everolimus Benefit in Hormone Receptor-Positive, Human Epidermal Growth Factor Receptor 2-Negative Advanced Breast Cancer: Results From BOLERO-2. *J Clin Oncol* (2016) 34:419–26. doi: 10.1200/JCO.2014.60.1971
26. Hyman DM, Smyth LM, Donoghue MTA, Chang MT, Reichel JB, Bouvier N, et al. AKT Inhibition in Solid Tumors With AKT1 Mutations. *J Clin Oncol* (2017) 35:2251–9. doi: 10.1200/JCO.2017.73.0143
27. Lee K-M, Guerrero-Zotano AL, Servetto A, Sudhan DR, Lin C-C, Formisano L, et al. Proline Rich 11 (PRR11) Overexpression Amplifies PI3K Signaling and Promotes Antiestrogen Resistance in Breast Cancer. *Nat Commun* (2020) 11:5488. doi: 10.1038/s41467-020-19291-x
28. Ellis MJ, Tao Y, Young O, White S, Proia AD, Murray J, et al. Estrogen-Independent Proliferation is Present in Estrogen-Receptor HER2-Positive Primary Breast Cancer After Neoadjuvant Letrozole. *J Clin Oncol* (2006) 24:3019–25. doi: 10.1200/JCO.2005.04.3034
29. Nayar U, Cohen O, Kapstad C, Cuoco MS, Waks AG, Wander SA, et al. Acquired HER2 Mutations in ER+ Metastatic Breast Cancer Confer Resistance to Estrogen Receptor–Directed Therapies. *Nat Genet* (2018) 51:207–16. doi: 10.1038/s41588-018-0287-5
30. Smyth LM, Piha-Paul SA, Won HH, Schram AM, Saura C, Loi S, et al. Efficacy and Determinants of Response to HER Kinase Inhibition in HER2-Mutant Metastatic Breast Cancer. *Cancer Discovery* (2020) 10:198–213. doi: 10.1158/2159-8290.CD-19-0966
31. Rasavi P, Chang MT, Xu G, Bandlamudi C, Ross DS, Vasan N, et al. The Genomic Landscape of Endocrine-Resistant Advanced Breast Cancers. *Cancer Cell* (2018) 34:427–438.e6. doi: 10.1016/J.CCELL.2018.08.008
32. Aftimos P, Oliveira M, Irrthum A, Fumagalli D, Sotiriou C, Gal-Yam EN, et al. Genomic and Transcriptomic Analyses of Breast Cancer Primaries and Matched Metastases in AURORA, the Breast International Group (BIG) Molecular Screening Initiative. *Cancer Discovery* (2021) 11:2796–811. doi: 10.1158/2159-8290.CD-20-1647
33. Bertucci F, Ng CKY, Patsouris A, Droin N, Piscuoglio S, Carbuccia N, et al. Genomic Characterization of Metastatic Breast Cancers. *Nature* (2019) 569:560–4. doi: 10.1038/s41586-019-1056-z
34. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 Ligand-Binding Domain Mutations in Hormone-Resistant Breast Cancer. *Nat Genet* (2013) 45:1439–45. doi: 10.1038/ng.2822
35. Toy W, Weir H, Razavi P, Lawson M, Goeppert AU, Mazzola AM, et al. Activating ESR1 Mutations Differentially Affect the Efficacy of ER Antagonists. *Cancer Discovery* (2017) 7:277–87. doi: 10.1158/2159-8290.CD-15-1523
36. Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, et al. Analysis of ESR1 Mutation in Circulating Tumor DNA Demonstrates Evolution During Therapy for Metastatic Breast Cancer. *Sci Trans Med* (2015) 7:1–9. doi: 10.1126/scitranslmed.aac7551
37. Jeselsohn R, Bergholz JS, Pun M, Cornwell M, Liu W, Nardone A, et al. Allele-Specific Chromatin Recruitment and Therapeutic Vulnerabilities of ESR1 Activating Mutations. *Cancer Cell* (2018) 33:173–186.e5. doi: 10.1016/J.CCELL.2018.01.004
38. Corces MR, Granja JM, Shams S, Louie BH, Seoane JA, Zhou W, et al. The Chromatin Accessibility Landscape of Primary Human Cancers. *Science* (2018) 362. doi: 10.1126/science.aav1898
39. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, et al. Genome-Wide Analysis of Estrogen Receptor Binding Sites. *Nat Genet* (2006) 38:1289–97. doi: 10.1038/ng1901
40. Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS. FOXA1 is a Key Determinant of Estrogen Receptor Function and Endocrine Response. *Nat Genet* (2011) 43:27–33. doi: 10.1038/ng.730
41. Gao S, Chen S, Han D, Wang Z, Li M, Han W, et al. Chromatin Binding of FOXA1 is Promoted by LSD1-Mediated Demethylation in Prostate Cancer. *Nat Genet* (2020) 52:1011–7. doi: 10.1038/s41588-020-0681-7
42. Xu G, Chhangawala S, Cocco E, Rasavi P, Cai Y, Otto JE, et al. ARID1A Determines Luminal Identity and Therapeutic Response in Estrogen-Receptor-Positive Breast Cancer. *Nat Genet* (2020) 52:198–207. doi: 10.1038/S41588-019-0554-0
43. Toska E, Osmanbeyoglu HU, Castel P, Chan C, Hendrickson RC, Elkabets M, et al. PI3K Pathway Regulates ER-Dependent Transcription in Breast Cancer Through the Epigenetic Regulator KMT2D. *Science* (2017) 355:1324–30. doi: 10.1126/science.aah6893
44. Arruabarrena-Aristorena A, Maag JLV, Kittane S, Cai Y, Karthaus WR, Ladewig E, et al. FOXA1 Mutations Reveal Distinct Chromatin Profiles and Influence Therapeutic Response in Breast Cancer. *Cancer Cell* (2020) 38:1–17. doi: 10.1016/j.ccell.2020.08.003
45. Rheinbay E, Parasuraman P, Grimsby J, Tiao G, Engreitz JM, Kim J, et al. Recurrent and Functional Regulatory Mutations in Breast Cancer. *Nature* (2017) 547:55–60. doi: 10.1038/nature22992
46. Fu X, Pereira R, de Angelis C, Veeraraghavan J, Nanda S, Qin L, et al. FOXA1 Upregulation Promotes Enhancer and Transcriptional Reprogramming in Endocrine-Resistant Breast Cancer. *Proc Natl Acad Sci* (2019) 116:26823–34. doi: 10.1073/PNAS.1911584116
47. Adams EJ, Karthaus WR, Hoover E, Liu D, Gruet A, Zhang Z, et al. FOXA1 Mutations Alter Pioneering Activity, Differentiation and Prostate Cancer Phenotypes. *Nature* (2019) 571:408–12. doi: 10.1038/s41586-019-1318-9
48. Parolia A, Cieslik M, Chu SC, Xiao L, Ouchi T, Zhang Y, et al. Distinct Structural Classes of Activating FOXA1 Alterations in Advanced Prostate Cancer. *Nature* (2019) 571:413–8. doi: 10.1038/s41586-019-1347-4
49. Jacobs J, Atkins M, Davie K, Imrichova H, Romanelli L, Christiaens V, et al. The Transcription Factor Grainy Head Primes Epithelial Enhancers for Spatiotemporal Activation by Displacing Nucleosomes. *Nat Genet* (2018) 50:1011–20. doi: 10.1038/s41588-018-0140-x
50. Chi D, Singhal H, Li L, Xiao T, Liu W, Pun M, et al. Estrogen Receptor Signaling is Reprogrammed During Breast Tumorigenesis. *Proc Natl Acad Sci* (2019) 116:11437–43. doi: 10.1073/PNAS.1819155116
51. Cocce KJ, Jasper JS, Desautels TK, Everett L, Wardell S, Westerling T, et al. The Lineage Determining Factor GRHL2 Collaborates With FOXA1 to Establish a Targetable Pathway in Endocrine Therapy-Resistant Breast Cancer. *Cell Rep* (2019) 29:889–903.e10. doi: 10.1016/J.CELREP.2019.09.032
52. Paltoglou S, Das R, Townley SL, Hickey TE, Tarulli GA, Coutinho I, et al. Novel Androgen Receptor Coregulator GRHL2 Exerts Both Oncogenic and Antimetastatic Functions in Prostate Cancer. *Cancer Res* (2017) 77:3417–30. doi: 10.1158/0008-5472.CAN-16-1616
53. Magnani L, Ballantyne EB, Zhang X, Lupien M. Genomic Pioneer Function Drives ERa Signaling Underlying Progression in Breast Cancer. *PloS Genet* (2011) 7:1002368. doi: 10.1371/journal.pgen.1002368
54. Magnani L, Patten DK, Nguyen VTM, Hong SP, Steel JH, Patel N, et al. The Pioneer Factor PBX1 is a Novel Driver of Metastatic Progression in ER'-Positive Breast Cancer. *Oncotarget* (2015) 6:21878–91. doi: 10.18632/oncotarget.4243
55. Ramberg H, Grytli HH, Nygard S, Wang W, Ögren O, Zhao S, et al. PBX3 is a Putative Biomarker of Aggressive Prostate Cancer. *Int J Cancer* (2016) 139:1810–20. doi: 10.1002/IJC.30220
56. Magnani L, Stoeck A, Zhang X, Lánczky A, Mirabella AC, Wang T-L, et al. Genome-Wide Reprogramming of the Chromatin Landscape Underlies Endocrine Therapy Resistance in Breast Cancer. *Proc Natl Acad Sci United States America* (2013) 110:E1490. doi: 10.1073/PNAS.1219992110
57. Jiang YZ, Yu KD, Zuo WJ, Peng WT, Shao ZM. GATA3 Mutations Define a Unique Subtype of Luminal-Like Breast Cancer With Improved Survival. *Cancer* (2014) 120:1329–37. doi: 10.1002/CNCR.28566

58. Afzaljavan F, Sadr AS, Savas S, Pasdar A. GATA3 Somatic Mutations are Associated With Clinicopathological Features and Expression Profile in TCGA Breast Cancer Patients. *Sci Rep* (2021) 11:1–13. doi: 10.1038/s41598-020-80680-9
59. Ellis MJ, Ding L, Shen D, Luo J, Suman VJ, Wallis JW, et al. Whole-Genome Analysis Informs Breast Cancer Response to Aromatase Inhibition. *Nature* (2012) 486:353–60. doi: 10.1038/NATURE11143
60. Gustin JP, Miller J, Farag M, Marc Rosen D, Thomas M, Scharpf RB, et al. GATA3 Frameshift Mutation Promotes Tumor Growth in Human Luminal Breast Cancer Cells and Induces Transcriptional Changes Seen in Primary GATA3 Mutant Breast Cancers. *Oncotarget* (2017) 8:103415–27. doi: 10.18632/oncotarget.21910
61. Wang Q, Li W, Liu XS, Carroll JS, Jänne OA, Keeton EK, et al. A Hierarchical Network of Transcription Factors Governs Androgen Receptor-Dependent Prostate Cancer Growth. *Mol Cell* (2007) 27:380–92. doi: 10.1016/j.molcel.2007.05.041
62. Vidal SJ, Rodriguez-Bravo V, Quinn SA, Rodriguez-Barrueco R, Lujambio A, Williams E, et al. A Targetable GATA2-IGF2 Axis Confers Aggressiveness in Lethal Prostate Cancer. *Cancer Cell* (2015) 27:223–39. doi: 10.1016/J.CCELL.2014.11.013
63. Jeselsohn R, Barry WT, Migliaccio I, Biagioni C, Zhao J, de Tribolet-Hardy J, et al. TransCONFIRM: Identification of a Genetic Signature of Response to Fulvestrant in Advanced Hormone Receptor Positive Breast Cancer. *Clin Cancer Res* (2016) 22:5755. doi: 10.1158/1078-0432.CCR-16-0148
64. Patten DK, Corleone G, Győrffy B, Perone Y, Slaven N, Barozzi I, et al. Enhancer Mapping Uncovers Phenotypic Heterogeneity and Evolution in Patients With Luminal Breast Cancer. *Nat Med* (2018) 24:1469–80. doi: 10.1038/s41591-018-0091-x
65. Dhanasekaran R, Deutzmann A, Mahauad-Fernandez WD, Hansen AS, Gouw AM, Felsher DW. The MYC Oncogene — the Grand Orchestrator of Cancer Growth and Immune Evasion. *Nat Rev Clin Oncol* (2021) 19:1–14. doi: 10.1038/s41571-021-00549-2
66. Chen R, Guo S, Yang C, Sun L, Zong B, Li K, et al. Although C–MYC Contributes to Tamoxifen Resistance, it Improves Cisplatin Sensitivity in ER −positive Breast Cancer. *Int J Oncol* (2020) 56:932–44. doi: 10.3892/IJO.2020.4987
67. Yu L, Wang L, Mao C, Duraki D, Kim JE, Huang R, et al. Estrogen-Independent Myc Overexpression Confers Endocrine Therapy Resistance on Breast Cancer Cells Expressing Erαy537s and Erαd538g Mutations. *Cancer Lett* (2019) 442:373–82. doi: 10.1016/J.CANLET.2018.10.041
68. Fiorito E, Sharma Y, Gilfillan S, Wang S, Singh SK, Satheesh SV, et al. CTCF Modulates Estrogen Receptor Function Through Specific Chromatin and Nuclear Matrix Interactions. *Nucleic Acids Res* (2016) 44:10588. doi: 10.1093/NAR/GKW785
69. Achinger-Kawecka J, Valdes-Mora F, Luu P-L, Giles KA, Caldon CE, Qu W, et al. Epigenetic Reprogramming at Estrogen-Receptor Binding Sites Alters 3D Chromatin Landscape in Endocrine-Resistant Breast Cancer. *Nat Commun* (2020) 11:1–17. doi: 10.1038/s41467-019-14098-x
70. Watt AC, Cejas P, DeCristo MJ, Metzger-Filho O, Lam EYN, Qiu X, et al. CDK4/6 Inhibition Reprograms the Breast Cancer Enhancer Landscape by Stimulating AP-1 Transcriptional Activity. *Nat Cancer* (2021) 2:34–48. doi: 10.1038/s43018-020-00135-y
71. Girault I, Lerebours F, Amarir S, Tozlu S, Tubiana-Hulin M, Lidereau R, et al. Expression Analysis of Estrogen Receptor α Coregulators in Breast Carcinoma: Evidence That NCOR1 Expression is Predictive of the Response to Tamoxifen. *Clin Cancer Res* (2003) 9:1259–66.
72. Bi M, Zhang Z, Jiang Y-Z, Xue P, Wang H, Lai Z, et al. Enhancer Reprogramming Driven by High-Order Assemblies of Transcription Factors Promotes Phenotypic Plasticity and Breast Cancer Endocrine Resistance. *Nat Cell Biol* (2020) 22:701–15. doi: 10.1038/s41556-020-0514-z
73. Mu P, Zhang Z, Benelli M, Karthaus WR, Hoover E, Chen CC, et al. SOX2 Promotes Lineage Plasticity and Antiandrogen Resistance in TP53- and RB1-Deficient Prostate Cancer. *Sci (New York NY)* (2017) 355:84–8. doi: 10.1126/SCIENCE.AAH4307
74. Gala K, Li Q, Sinha A, Razavi P, Dorso M, Sanchez-Vega F, et al. KMT2C Mediates the Estrogen Dependence of Breast Cancer Through Regulation of Erα Enhancer Function. *Oncogene* (2018) 37:4692–710. doi: 10.1038/S41388-018-0273-5
75. Yamamoto S, Wu Z, Russnes HG, Takagi S, Peluffo G, Vaske C, et al. Article JARID1B Is a Luminal Lineage-Driving Oncogene in Breast Cancer. *Cancer Cell* (2014) 25:762–77. doi: 10.1016/j.ccr.2014.04.024
76. Hinohara K, Wu H-J, Vigneau S, McDonald TO, Igarashi KJ, Yamamoto KN, et al. KDM5 Histone Demethylase Activity Links Cellular Transcriptomic Heterogeneity to Therapeutic Resistance. *Cancer Cell* (2018) 34:939–953.e9. doi: 10.1016/J.CCELL.2018.10.014
77. Bosch A, Li Z, Bergamaschi A, Ellis H, Toska E, Prat A, et al. PI3K Inhibition Results in Enhanced Estrogen Receptor Function and Dependence in Hormone Receptor-Positive Breast Cancer. *Sci Trans Med* (2015) 7 (283):283ra51. doi: 10.1126/SCITRANSLMED.AAA4442
78. Toska E, Castel P, Chhangawala S, Arruabarrena-Aristorena A, Chan C, Hristidis VC, et al. PI3K Inhibition Activates SGK1 *via* a Feedback Loop to Promote Chromatin-Based Regulation of ER-Dependent Gene Expression. *Cell Rep* (2019) 27:294–306. doi: 10.1016/j.celrep.2019.02.111
79. Vanhaesebroeck B, Perry MWD, Brown JR, André F, Okkenhaug K. PI3K Inhibitors are Finally Coming of Age. *Nat Rev Drug Discovery* (2021) 20:741–69. doi: 10.1038/S41573-021-00209-1
80. Castel P, Toska E, Engelman JA, Scaltriti M. The Present and Future of PI3K Inhibitors for Cancer Therapy. *Nat Cancer 2021 2:6* (2021) 2:587–97. doi: 10.1038/s43018-021-00218-4
81. Vasan N, Cantley LC. At a Crossroads: How to Translate the Roles of PI3K Oncogenic and Metabolic Signalling Into Improvements for Cancer Therapy. *Nat Rev Clin Oncol* (2022) doi: 10.1038/s41571-022-00633-1
82. Pearson A, Proszek P, Pascual J, Fribbens C, Shamsher MK, Kingston B, et al. Inactivating NF1 Mutations Are Enriched in Advanced Breast Cancer and Contribute to Endocrine Therapy Resistance. *Clin Cancer Res* (2020) 26:608–22. doi: 10.1158/1078-0432.CCR-18-4044
83. Servetto A, Kollipara R, Formisano L, Lin C-C, Lee K-M, Sudhan DR, et al. Nuclear FGFR1 Regulates Gene Transcription and Promotes Antiestrogen Resistance in ER+ Breast Cancer. *Clin Cancer Res* (2021) 27:4379–96. doi: 10.1158/1078-0432.CCR-20-3905
84. Mohammed H, Russell IA, Stark R, Rueda OM, Hickey TE, Tarulli GA, et al. Progesterone Receptor Modulates Erα Action in Breast Cancer. *Nature* (2015) 523:313–7. doi: 10.1038/nature14583
85. D'Amato NC, Gordon MA, Babbs B, Spoelstra NS, Carson Butterfield KT, Torkko KC, et al. Cooperative Dynamics of AR and ER Activity in Breast Cancer. *Mol Cancer Res* (2016) 14:1054–67. doi: 10.1158/1541-7786.MCR-16-0167
86. Yang F, Ma Q, Liu Z, Li W, Tan Y, Jin C, et al. Glucocorticoid Receptor: MegaTrans Switching Mediates the Repression of an Erα-Regulated Transcriptional Program. *Mol Cell* (2017) 66:321–331.e6. doi: 10.1016/j.molcel.2017.03.019
87. Jhaveri K, Winer EP, Lim E, Fidalgo JA, Bellet M, Mayer IA, et al. Abstract PD7-05: A First-in-Human Phase I Study to Evaluate the Oral Selective Estrogen Receptor Degrader (SERD), GDC-9545, in Postmenopausal Women With Estrogen Receptor-Positive (ER<sup<+</sup<) HER2-Negative (HER2<sup<-≪/Sup<) M. *Cancer Res* (2020) 80:PD7–05. doi: 10.1158/1538-7445.SABCS19-PD7-05
88. Kaklamani V, Bardia A, Wilks S, Weise A, Richards D, Harb W, et al. Abstract PD7-07: Final Analysis of Phase 1 Study of Elacestrant (RAD1901), a Novel Selective Estrogen Receptor Degrader (SERD), in Estrogen Receptor Positive (ER+), Human Epidermal Growth Factor Receptor 2 Negative (HER2-) Advanced Breast Cancer. *Cancer Res* (2020) 80:PD7–07. doi: 10.1158/1538-7445.SABCS19-PD7-07
89. Paoletti C, Schiavon G, Dolce EM, Darga EP, Carr TH, Geradts J, et al. Circulating Biomarkers and Resistance to Endocrine Therapy in Metastatic Breast Cancers: Correlative Results From AZD9496 Oral SERD Phase I Trial. *Clin Cancer Res* (2018) 24:5860–5872. doi: 10.1158/1078-0432.CCR-18-1569
90. Qi S-M, Dong J, Xu Z-Y, Cheng X-D, Zhang W-D, Qin J-J. PROTAC: An Effective Targeted Protein Degradation Strategy for Cancer Therapy. *Front Pharmacol* (2021) 12:1124. doi: 10.3389/fphar.2021.692574
91. Nagarajan S, Rao SV, Sutton J, Cheeseman D, Dunn S, Papachristou EK, et al. ARID1A Influences HDAC1/BRD4 Activity, Intrinsic Proliferative Capacity and Breast Cancer Treatment Response. *Nat Genet* (2020) 52:187–97. doi: 10.1038/S41588-019-0541-5
92. Bitler BG, Aird KM, Garipov A, Li H, Amatangelo M, Kossenkov AV, et al. Synthetic Lethality by Targeting EZH2 Methyltransferase Activity in ARID1A-Mutated Cancers. *Nat Med* (2015) 21:231–8. doi: 10.1038/NM.3799

93. Sharma MR, Carvajal RD, Catenacci D, Emens LA, Hanna GJ, Juric D, et al. Abstract CT218: Phase 1/2 Study of a Novel HER2 Targeting TLR7/8 Immune-Stimulating Antibody Conjugate (ISAC), BDC-1001, Alone and in Combination With Pembrolizumab (Pembro) in Patients (Pts) With HER2-Expressing Advanced Solid Tumors. *Cancer Res* (2021) 81:CT218. doi: 10.1158/1538-7445.AM2021-CT218
94. Zehir A, Benayed R, Shah RH, Syed A, Middha S, Kim HR, et al. Mutational Landscape of Metastatic Cancer Revealed From Prospective Clinical Sequencing of 10,000 Patients. *Nat Med* (2017) 23:703–13. doi: 10.1038/NM.4333
95. Cheng DT, Mitchell TN, Zehir A, Shah RH, Benayed R, Syed A, et al. Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT): A Hybridization Capture-Based Next-Generation Sequencing Clinical Assay for Solid Tumor Molecular Oncology. *J Mol Diagnostics* (2015) 17:251. doi: 10.1016/J.JMOLDX.2014.12.006

Conflict of Interest: ET has received honoraria, consulting fees, and a grant from AstraZeneca.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 12 August 2022
DOI 10.3389/fonc.2022.913072

OPEN ACCESS

EDITED BY
Rita G. Nunes,
Universidade de Lisboa, Portugal

REVIEWED BY
Minna Sikiö,
Tampere University Hospital, Finland
Murat Baykara,
Firat University, Turkey

*CORRESPONDENCE
Kun Sun
sk12177@rjh.com.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 05 April 2022
ACCEPTED 13 July 2022
PUBLISHED 12 August 2022

CITATION
Sun K, Zhu H, Xia B, Li X, Chai W, Fu C, Thomas B, Liu W, Grimm R, Elisabeth W and Yan F (2022) Image quality and whole-lesion histogram and texture analysis of diffusion-weighted imaging of breast MRI based on advanced ZOOMit and simultaneous multislice readout-segmented echo-planar imaging. *Front. Oncol.* 12:913072. doi: 10.3389/fonc.2022.913072

Image quality and whole-lesion histogram and texture analysis of diffusion-weighted imaging of breast MRI based on advanced ZOOMit and simultaneous multislice readout-segmented echo-planar imaging

Kun Sun[1*†], Hong Zhu[1†], Bingqing Xia[2], Xinyue Li[3], Weimin Chai[1], Caixia Fu[4], Benkert Thomas[5], Wei Liu[4], Robert Grimm[5], Weiland Elisabeth[5] and Fuhua Yan[1]

[1]Department of Radiology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China, [2]Department of Radiology, International Peace Maternity and Child Health Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China, [3]Department of Radiology, Ruijin Hospital Luwan Branch, Shanghai Jiaotong University School of Medicine, Shanghai, China, [4]MR Application Development, Siemens Shenzhen Magnetic Resonance Ltd., Shenzhen, China, [5]MR Application Predevelopment, Siemens Healthcare, Erlangen, Germany

Objectives: To investigate the image quality and diagnostic capability a of whole-lesion histogram and texture analysis of advanced ZOOMit (A-ZOOMit) and simultaneous multislice readout-segmented echo-planar imaging (SMS-RS-EPI) to differentiate benign from malignant breast lesions.

Study design: From February 2020 to October 2020, diffusion-weighted imaging (DWI) using SMS-RS-EPI and A-ZOOMit were performed on 167 patients. Three breast radiologists independently ranked the image datasets. The inter-/intracorrelation coefficients (ICCs) of mean image quality scores and lesion conspicuity scores were calculated between these three readers. Histogram and texture features were extracted from the apparent diffusion coefficient (ADC) maps, respectively, based on a WL analysis. Student's t-tests, one-way ANOVAs, Mann–Whitney U tests, and receiver operating characteristic curves were used for statistical analysis.

Results: The overall image quality scores and lesion conspicuity scores for A-ZOOMit and SMS-RS-EPI showed statistically significant differences (4.92 $\pm$ 0.27 *vs.* 3.92 $\pm$ 0.42 and 4.93 $\pm$ 0.29 *vs.* 3.87 $\pm$ 0.47, $p < 0.0001$). The ICCs for the image quality and lesion conspicuity scores had good agreements among the three readers (all ICCs >0.75). To differentiate benign and malignant breast lesions, the entropy of $ADC_{A\text{-}Zoomit}$ had the highest area (0.78) under the ROC curve.

Conclusions: A-ZOOMit achieved higher image quality and lesion conspicuity than SMS-RS-EPI. Entropy based on A-ZOOMit is recommended for differentiating benign from malignant breast lesions.

KEYWORDS

breast neoplasm, magnetic resonance imaging, diffusion weighted imaging, whole lesion, histogram analysis, texture analysis

Introduction

Breast MRI is the most sensitive imaging modality for the evaluation of breast cancer detection, diagnosis, and prognosis (1, 2). Diffusion-weighted imaging (DWI) can be used as an adjunct sequence to dynamic contrast-enhanced MRI (3–6).

Readout-segmented echo-planar imaging (RS-EPI) (7–9) with shortened echo spacing and echo train length (ETL), is associated with less geometric distortions and higher spatial resolution than single short echo-planar imaging (SS-EPI). However, the scan time of RS-EPI is significantly longer than that of SS-EPI. Simultaneous multislice excitation technique (SMS) allows one to acquire several slices in parallel so that fewer slice excitations are required to achieve the same slice coverage (10–12), thus improving the acquisition speed of RS-EPI DWI (13, 14).

The zoomed technique uses 2D radio-frequency pulses to excite a small field of view (FOV) in the phase-encoding direction thus shortening the readout ETL, with improved resolution, less geometric distortions, and less susceptibility artifact (15). The conventional zoomed technique is often associated with aliasing artifacts in the FOV due to the discretized sampling of the excitation k-space with resulting trajectory errors that lead to side excitations. Finsterbuch (16) proposed that a slight rotation of the field of excitation when performing zoomed EPI can mitigate potential aliasing artifacts, in the following referred to advanced ZOOMit (A-ZOOMit). Zoomed EPI DWI has been used on the prostate and other body regions (15, 17, 18). Equipped with complex-averaging and rigid motion registration among different b values and measurements, A-ZOOMit could achieve more excellent image quality and lesion conspicuity (18). Furthermore, no study has been published on breast tumors with the use of A-ZOOMit.

Whole-lesion (WL) histogram and texture analysis (19–21) show the probability distributions of continuous variables and the spatial distributions of gray values, which provides information about tumor heterogeneity. Previous studies (19, 22–24) show that histogram and texture analysis can achieve higher diagnostic accuracy compared with the use of only the mean values of parameters.

Hence, the objective of this study was to explore the image quality and feasibility of the A-ZOOMit and SMS-RS-EPI in clinical practice and to investigate the diagnostic capabilities of the WL histogram and texture analysis of A-ZOOMit and SMS-RS-EPI for further characterization of breast lesions.

Abbreviations: AUC, area under the receiver operating characteristic curve; BI-RADS, breast imaging reporting and data system; ROC, receiver operating characteristic; WL, whole lesion; DWI, diffusion-weighted imaging; SMS, simultaneous multislice; A-ZOOMit, advanced ZOOMit; RS-EPI, readout segmented echo-planar imaging; SS-EPI, single-shot echo-planar imaging; EPI, echo-planar imaging; ETL, echo train length; ADC, apparent diffusion coefficient; FOV, field of view; ICCs, inter-/intracorrelation coefficients; CI, confidence interval; SNR, signal-to-noise ratio; CNR, contrast-to-noise ratio. ; MRI, magnetic resonance imaging; ROI, region of interest; ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human epidermal growth factor receptor-2.

Materials and methods

Study population

The local institutional review board approved this study. We obtained written informed consent from all participants. From February 2020 to October 2020, we enrolled 197 women with lesions suspicious for breast cancer on mammography or ultrasonography [i.e., Breast Imaging Reporting and Data System (BI-RADS) categories 4 or 5] who underwent SMS-RS-EPI and A-ZOOMit examinations. The exclusion criteria included the following: patients previously treated for a malignancy (n =10), patients without histopathological results (n = 5), patients with motion artifact (n =10), and patients with no lesion shown in DWI (n =5). For the 13 patients with multicentric or multifocal tumors, lesions with the largest sizes according to the postcontrast images were analyzed. Ultimately, 167 women (mean age, 53 years; age range, 22–82 years), with 167 lesions (mean size, 2.1 cm; range, 0.4–5.7 cm) were enrolled in the study.

Magnetic resonance imaging scanning

All breast MRI examinations were performed on a 1.5-T system (MAGNETOM Aera; Siemens Healthcare, Erlangen, Germany) with a dedicated 18-channel phased-array breast coil. Before DCE-MRI, we performed axial bilateral fat-suppressed T2-weighted fast spin-echo imaging, prototype SMS-RS-EPI, and prototype A-ZOOMit on each patient. A-ZOOMit was performed with a slight rotation of the field of excitation, motion registration, and complex averaging, The other parameters of SMS-RS-EPI and A-ZOOMit are shown in Table 1. Apparent diffusion coefficient (ADC) maps were online-generated for the two DWI sequences.

Multireader evaluation of image quality and lesion conspicuity scores

Three breast radiologists, each from a different hospital, independently evaluated and scored the overall image quality and lesion conspicuity of the SMS-RS-EPI and A-ZOOMit images (SK with 9 years of experience, XBQ with 6 years of experience, and LXY with 5 years of experience; **. performed the image quality measurement twice to calculate the intraclass agreement). All three radiologists were blinded to the sequence type when they evaluated the image quality and lesion conspicuity. The radiologists scored the overall image quality on a 5-point quality scale (1 = nondiagnostic, 2 = limited, 3 = diagnostic, 4 = good, and 5 = excellent). The radiologists scored lesion conspicuity on a scale of 1 (lesion not visible) to 5 (excellent visibility).

TABLE 1 Sequence parameters for advanced ZOOMit (A-ZOOMit) and simultaneous multislice readout segmented echo-planar imaging (SMS-RS-EPI DWI).

Sequence Parameter	A-ZOOMit	SMS-RS-EPI
Diffusion mode	3D diagonal	3D diagonal
b values (s/mm^2)	0,1,000	0,1,000
Average	b_0 (7), $b_{1,000}$ (21)	b_0 (2), $b_{1,000}$ (6)
Repetition time (ms)	5,700	3,780
Echo time	83	78
Orientation	Transversal	Transversal
FOV (mm^2)	340*158	340*155
Scan matrix	220*102	220*100
Slice thickness (mm)	4	4
Slices	26	26
Readout segments	1	5
Oversampling in PE dir.	0	50%
No. of Sat.band	0	2
Fat suppression	SPAIR	SPAIR
Voxel size	1.5*1.5*4	1.5*1.5*4
Acquisition time	2:57	3:01
Bandwidth (Hz/Px)	988	668
Accel.factor PE	2	2
Accel factor slice	1	2
PE dir.	P ≥ A	P ≥ A

SMS-RS-EPI; simultaneous multislice (SMS) readout segmented echo-planar imaging; FOV, field of view; Px, pixel; PE, phase encoding. Sat. band, saturation band, which was used to suppress the signal from the back, to avoid the aliasing artifact; P, posterior; A, anterior; dir., direction.

The signal-to-noise ratio (SNR) and contrast-to-noise ratio (CNR) of the lesions on DWI with $b_{1,000}$ were also evaluated. SNR was defined as SNR = $S_{Lesion}/\sigma_{noise}$, where S_{Lesion} is the mean signal intensity of an region of interest (ROI) within the lesion, and σ_{noise} is the standard deviation (SD) of the background noise. CNR was defined as CNR = $(S_{Lesion} - S_{Tissue})/\sigma_{noise}$, where S_{Tissue} is the mean signal intensity of an ROI on the normal breast tissue.

Histogram and texture analysis based on apparent diffusion coefficient maps

Histogram and texture analyses for the SMS-RS-EPI- and A-ZOOMit-derived ADC maps were performed on the prototype MR Multiparametric Analysis software (Siemens Healthcare, Erlangen, Germany) by radiologists (**. and **) with four steps (19, 25). The four steps of data analysis were as follows:

1. Data loading: The ADC maps and b value of 1,000 images of both SMS-RESOLVE and A-ZOOMit were loaded to the software.
2. Seed point drawing: For ADC map analysis, foreground and background seed points were manually drawn inside and outside of the tumor on the three multiplanar reconstruction planes of the $b_{1,000}$ images of SMS-RESOLVE.
3. Segmentation: The whole tumor was segmented by the software based on the seed points using a random walker algorithm. Manual adjustments were performed if the initial segmentation result was not satisfactory. The final three-dimensional (3D)-segmented volumes that were created on the $b_{1,000}$ images were then automatically propagated to the A-ZOOMit maps.
4. Histogram and texture analyses: The WL histogram and texture analyses on the parametric maps were automatically performed by a one-push button. A total of seven histogram-based statistical features and four texture-based features were extracted. Histogram-based features included mean, SD, median, percentiles (5th and 95th), skewness (measure of asymmetry of the probability distribution), and kurtosis (measure of the shape of the probability distribution). Texture-based features included entropy (measure of the randomness of the gray levels), contrast (measure of the amount of

gray-level variations), difference entropy (diff-entropy, measure of the entropy difference), and difference variance (diff-variance, measure of variation in the difference in gray levels between voxel pairs).

Histopathologic analysis

The hematoxylin and eosin staining results and the immunohistochemical analysis of surgical specimens were reviewed in every patient's medical record. Axillary lymph node metastasis; the expression status of Estrogen receptor (ER), Progesterone receptor (PR), and Human epidermal growth factor receptor-2 (HER2); and Ki-67 were routinely recorded.

Statistical analysis

We described the clinical characteristics using frequencies for categorical

variables and means and ranges for all continuous variables. We compared the differences in clinical characteristics using chi-square tests and the analysis of variance. We used the Student's t-test or one-way ANOVA in univariate analyses when the data were normally distributed and the Mann–Whitney U test when the data were not normally distributed.

We calculated the average scores from the three readers' measurements and then used the Wilcoxon signed-rank tests to determine if differences existed between scores. We evaluated the intra- and interclass agreement of the readers' scores. Then, we calculated the intra- and interclass agreement among the readers' scores. The intraclass correlation coefficient (ICC_{intra}) was computed from radiologist 1's two measurements. The interclass correlation coefficients were computed between radiologist 1's first measurements and radiologist 2's and radiologist 3's measurements ($ICC_{1,2}$ and $ICC_{1,3}$, respectively). We interpreted an ICC greater than 0.75 as indicative of good agreement.

We used SPSS (v. 26.0; SPSS, Chicago, IL, USA) and MedCalc (MedCalc, Mariakerke, Belgium) for the statistical analyses. We considered a *p*-value less than 0.05 indicative of statistically significant difference.

Results

Clinical characteristics

There were significant differences in demographic characteristics between patients with malignant lesions (mean age, 55.0 ± 11.7 years; range, 28–81 years) and patients with benign lesions (mean age, 48.5 ± 11.7 years; range, 22–82 years; $p = 0.001$).

Pathological features

Of the 167 lesions, 110 were malignant, and 57 were benign. There was a significant difference in the lesion size between malignant and benign breast lesions (2.27 ± 0.94 cm *vs.* 1.71 ± 0.98 cm, $p < 0.0001$).

The malignant lesions included ductal carcinoma *in situ* (N = 18), invasive carcinoma of no special type (N = 87), invasive lobular carcinoma (N = 1), invasive solid papillary carcinoma (N = 1), mucinous carcinoma (N = 2), and encapsulated papillary carcinoma with invasion (N = 1). Of the 92 invasive breast cancers in this study, 21 (22.8%) were luminal A cancer, 30 (32.6%) were luminal B cancer, 33 (35.9%) were HER2-positive cancer, and 8 (8.7%) were triple-negative cancer. Of all these invasive cancers, there were 61 (66%) patients who were lymph node–negative, and there were 31 (34%) patients who were lymph node–positive.

Benign lesions included fibroadenoma (N = 24), benign phyllodes tumors (N = 1), fibrocystic change (N = 7), cyst-combined chronic infection (N = 9), papilloma (N = 8), usual ductal hyperplasia (N = 4), and adenosis (N = 4).

Image quality score comparisons of SMS-RS-EPI and A-ZOOMit

The mean overall image quality scores of A-ZOOMit and SMS-RS-EPI showed a statistically significant difference (4.92 ± 0.27 *vs.* 3.92 ± 0.42, $p < 0.0001$, respectively, in the multireader study).

For the image quality score of A-ZOOMit images, the ICC_{intra} was 0.94, $ICC_{1,2}$ was 0.79, $ICC_{2,3}$ was 0.80, and $ICC_{1,3}$ was 0.85. For the SMS-RS-EPI, the ICC_{intra} was 0.92, $ICC_{1,2}$ was 0.85, $ICC_{2,3}$ was 0.77, and $ICC_{1,3}$ was 0.75. The details of ICCs are shown in Table 2. A case of $b_{1,000}$ based on SMS-RESOLVE and A-ZOOMit is shown in Figure 1.

Lesion conspicuity score comparisons of SMS-RS-EPI and A-ZOOMit

The mean lesion conspicuity scores of the A-ZOOMit and SMS-RS-EPI showed a significant difference (4.93 ± 0.29 *vs.* 3.87 ± 0.47, $p < 0.0001$, respectively, in the multireader study).

For the lesion conspicuity score of A-ZOOMit images, the ICC_{intra} was 0.94, the $ICC_{1,2}$ was 0.77, the $ICC_{2,3}$ was 0.83, and the $ICC_{1,3}$ was 0.75. For the SMS-RS-EPI, the ICC_{intra} was 0.90, the $ICC_{1,2}$ was 0.83, the $ICC_{2,3}$ was 0.81, and the $ICC_{1,3}$ was 0.78.

TABLE 2 Intra- and interclass correlation coefficients of multireader ratings of image-quality and lesion conspicuity on A-ZOOMit and SMS-RS-EPI.

	Radiologist 1	Radiologist 3
A-ZOOMit		
Image Quality		
Radiologist 1	0.94 (0.84–0.91)	0.75 (0.50–0.99)
Radiologist 2	0.79 (0.60–0.97)	0.83 (0.68–0.98)
Lesion Conspicuity		
Radiologist 1	0.90 (0.87–0.93)	0.80 (0.71–0.89)
Radiologist 2	0.83 (0.71–0.96)	0.80 (0.70–0.90)
SMS-RS-EPI		
Image Quality		
Radiologist 1	0.92 (0.90–0.94)	0.78 (0.66–0.89)
Radiologist 2	0.85 (0.69–1.0)	0.77 (0.63–0.91)
Lesion Conspicuity		
Radiologist 1	0.86 (0.82–0.89)	0.80 (0.71–0.89)
Radiologist 2	0.80 (0.69–0.90)	0.81 (0.70–0.92)

Data in parentheses represent the 95% confidence interval. SMS-RS-EPI; simultaneous multislice (SMS) readout-segmented echo-planar imaging.

Histogram and texture analyses of ADC in differentiating malignant and benign breast lesions

The histogram and texture features to distinguish between malignant and benign breast lesions are shown in Table 3 and Supplementary Table 1. Imaging examples are shown in Figures 2, 3.

The mean, median, and 5th percentile of the ADCs based on A-ZOOMit were significantly lower in the malignant lesions than in the benign tumors (p < 0.0001, < 0.0001, 0.011, respectively). However, the skewness, entropy, and diff-entropy of the ADCs based on A-ZOOMit were significantly higher in the malignant lesions than in the benign tumors (p < 0.0001, < 0.0001, < 0.0001, respectively).

The mean and median value of the ADCs based on SMS-RS-EPI were significantly lower in the malignant lesions than in the benign tumors (p = 0.008, 0.001, respectively). However, the skewness, entropy, and diff-entropy of the ADCs based on SMS-RS-EPI were significantly higher in the malignant lesions than in the benign tumors (p < 0.0001, < 0.0001, < 0.0001, respectively).

Differences of SNR and CNR between SMS-RS-EPI and A-ZOOMit

There was a significant difference of the $SNR_{b1,000}$ between the SMS-RS-EPI and A-ZOOMit images (30.62 ± 16.95 *vs.* 58.19 ± 33.34, p < 0.0001). Furthermore, the $CNR_{b1,000}$ based on SMS-RS-EPI and A-ZOOMit also shown significant difference (19.99 ± 15.10 *vs.* 37.42 ± 28.12, p < 0.0001 Figure 4).

Results of the receiver operating characteristic curves

To differentiate the benign from malignant breast lesions, the entropy value of ADC (0.78, 95% CI 0.71–0.84) from the A-ZOOMit texture analysis had the highest area under the ROC curve (Figure 5). The details of areas under the ROC curves are shown in Supplementary Table 2.

Discussion

In our study, we found a significantly higher image quality and lesion conspicuity of breast DWI based on A-ZOOMit, with

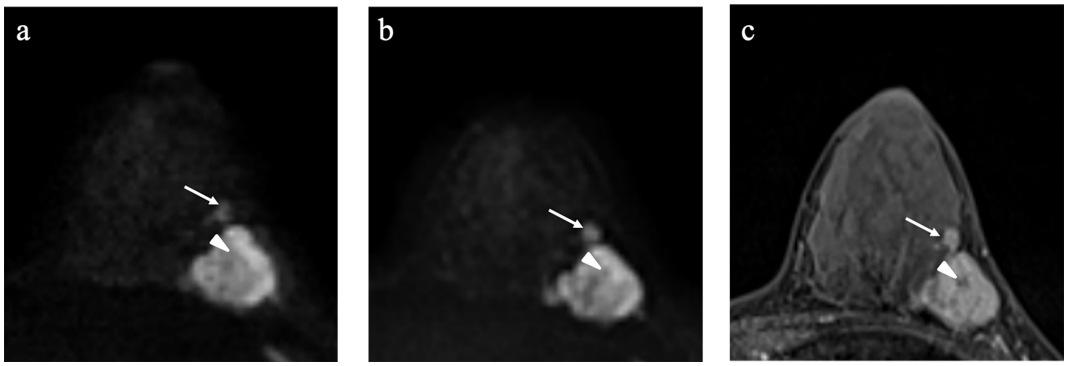


FIGURE 1
Example images of a 35-year-old woman with invasive ductal carcinoma in the left breast **(A–C)**. SMS-RS-EPI image of $b_{1,000}$ **(A)**; advanced ZOOMit (A-ZOOMit) image of $b_{1,000}$ **(B)**; dynamic contrast imaging of T1WI **(C)**. A-ZOOMit image showed a better image quality of the satellite nodule (long arrow) and necrosis (short arrow) than the SMS-RS-EPI image.

TABLE 3 Histogram and texture analysis of apparent diffusion coefficient values based on SMS-RS-EPI and A-ZOOMit between malignant and benign breast lesions.

Characteristics	Benign Lesions	Malignant Lesions	*p*-values
A-ZOOMit			
Mean	1.29 ± 0.28	1.14 ± 0.23	<;0.0001*
Median	1.30 ± 0.29	1.12 ± 0.25	<;0.0001*
5th percentile	0.63 ± 0.31	0.52 ± 0.21	0.011
Skewness	-0.18 ± 0.54	0.21 ± 0.56	<;0.0001*
Diff-entropy	2.11± 0.18	2.27 ± 0.19	<;0.0001*
Entropy	3.09 ± 0.21	3.27± 0.19	<;0.0001*
SMS-RS-EPI			
Mean	1.18 ± 0.28	1.07± 0.22	0.008
Median	1.20 ± 0.32	1.05 ± 0.24	0.001*
Skewness	-0.26 ± 0.57	0.20 ± 0.51	<;0.0001*
Diff-entropy	2.15 ± 0.19	2.25 ± 0.13	<;0.0001*
Entropy	3.13 ± 0.25	3.29 ± 0.12	<;0.0001*

SMS-RS-EPI; simultaneous multislice (SMS) readout-segmented echo-planar imaging; SD, standard deviation. * symbol represent significant difference.

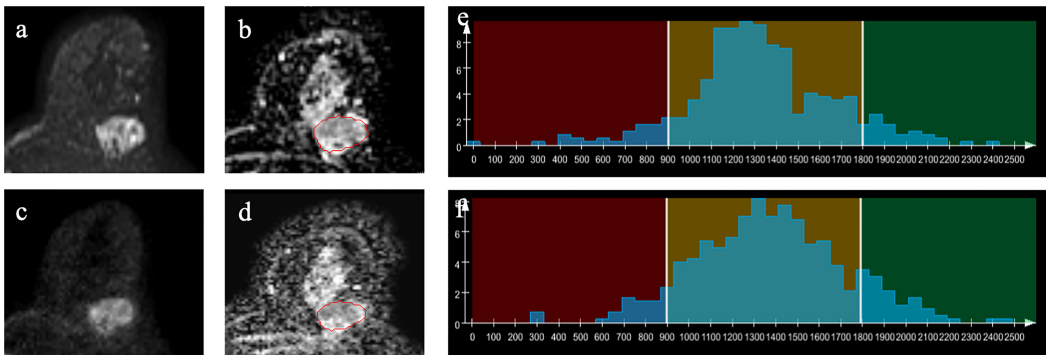


FIGURE 2
Example images of a 60-year-old woman with invasive ductal carcinoma in the left breast **(A–D)**. A-ZOOMit image of $b_{1,000}$ **(A)**; apparent diffusion coefficient (ADC) map based on A-ZOOMit **(B)**; SMS-RS-EPI image of $b_{1,000}$ **(C)**; ADC map based on SMS-RS-EPI **(D)**; histogram of segmented tumors based on ADC maps **(E, F)**.

an improved diagnostic performance of texture parameter entropy for differentiating between malignant and benign breast lesions, compared with SMS-RS-EPI.

Previous studies (15, 17, 18) using zoomed EPI were based on the conventional ZOOMit method. In our study, we used advanced ZOOMit, which could be able to further improve the image quality and the accuracy of the ADC estimation. SMS-RS-EPI has already been used in breast cancer diagnosis (14, 26, 27). Hu et al. (27) found that SMS-RS-EPI can significantly reduce the acquisition time and achieve a comparable diagnostic accuracy for the differentiation between malignant and benign breast lesions. In our study, we used identical scan time and the same b values for both A-ZOOMit and SMS-RS-EPI. We wanted to know which sequence would be the most preferred DWI sequence for the breast radiologists on a 1.5-T MRI scanner.

Our results showed that both the image quality and lesion conspicuity based on A-ZOOMit were higher than with SMS-RS-EPI in multireader studies. Furthermore, the appearance of the tumor details shown on A-ZOOMit were more suitable for radiologists' reading habits. The quantitative evaluation demonstrated that the SNR and CNR of the lesion on A-ZOOMit were also higher than that of SMS-RS-EPI. The better image quality, lesion conspicuity, and higher SNR and CNR of A-ZOOMit can be attributed to the higher number of averaging, the complex averaging scheme, and motion registration. As the zoomed FOV technique was used, neither oversampling in phase-encoding direction nor the saturation band on the back region were needed to avoid the aliasing artifacts for A-ZOOMit; thus, the scan time can be saved, and a higher number of averaging can be used compared to SMS-RS-

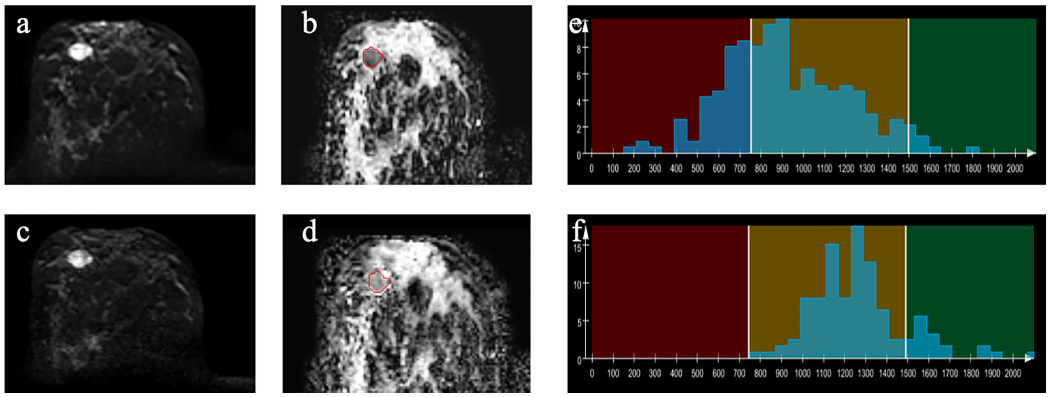


FIGURE 3
Example images of a 44-year-old woman with papilloma in the right breast **(A–D)**. A-ZOOMit image of $b_{1,000}$(**a**); ADC map based on A-ZOOMit **(B)**; SMS-RS-EPI image of $b_{1,000}$ **(C)**; ADC map based on SMS-RS-EPI **(D)**; histogram of segmented tumors based on ADC maps **(E, F)**.

EPI. Considering the benefits mentioned above, we recommended A-ZOOMit DWI for clinical breast application on a 1.5-T MRI scanner.

Our results show that most of the histogram and texture features of ADC can be used for the diagnosis of breast cancer. Suo et al. (28) also found that the entropy of ADC provided complementary information for evaluating IDC phenotypes. Additionally, in our study, the entropy of ADC based on A-ZOOMit showed the highest area under the ROC curves for the diagnosis of breast cancer, which was consistent with their study. Higher entropy represents higher cellular heterogeneity. Cellular heterogeneity among breast cancers may correlate with the histopathological changes of the hormone receptor status and HER2 status (29, 30). In our study, the HER2-positive cancer counts for 35.9%, which will be shown as a higher entropy value.

Our study had several limitations. First of all, the limited sample size and the imbalanced distribution of benign lesions. Second, we generated and analyzed only 11 commonly used texture features proved valuable in previous clinical

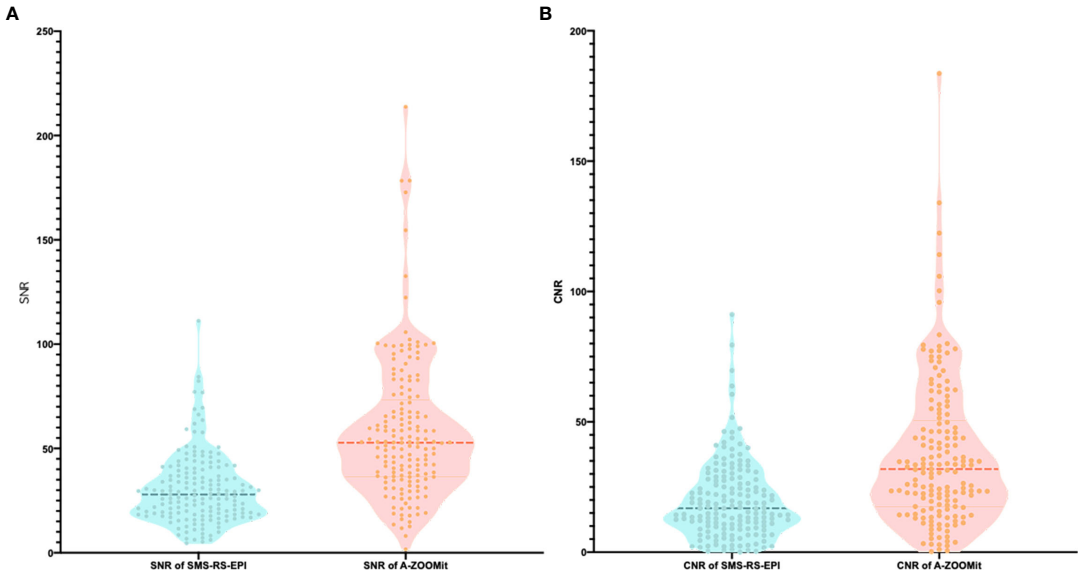


FIGURE 4
The SNR and CNR of b1,000 based on SMS-RS-EPI and A-ZOOMit. **(A, B)** There was a significant difference of SNR of b1000 based on SMS-RESOLVE and A-ZOOMit ($p < 0.001$) **(A)**; There was a significant difference of SNR of b1000 based on SMS RESOLVE and A-ZOOMit ($p < 0.001$) **(B)**.

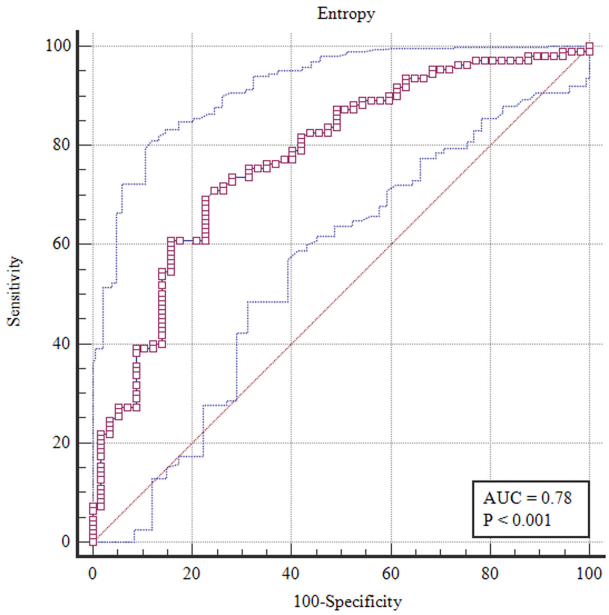


FIGURE 5
Receiver operating characteristic curve of entropy with 95% confidence interval based on A-ZOOMit for the differentiation between benign and malignant lesions.

applications (31). In addition, anisotropic voxel resolutions in our DWI data may not allow fully appreciating the 3D textural structure of the lesions. In our further study, we will enroll more high-order features to reflect tumor heterogeneity. Third, we used the background noise to estimate the noise level of the lesions for the calculation of SNR and CNR since the individual images were not available. This method is not suitable for estimating the noise when parallel imaging acceleration is used. In our future studies, we will utilize the method described by Reeder SB et al. (32). Fourth, we compared the two newly developed DWI sequences to each other and did not compare them with conventional SS-EPI DWI and/or RS-EPI sequences since many studies (7, 14, 33) have already compared them. Further large-scale multicenter studies could provide an evidence of the effect of advanced DWI methods on diagnostic accuracy.

We concluded that DWI based on A-ZOOMit provides significantly higher image quality and lesion conspicuity than SMS-RS-EPI in our study. Thereby, texture analysis based on A-ZOOMit achieved higher diagnostic accuracy for the differentiation of benign and malignant breast lesions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was reviewed and approved by Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Literature search: KS, and HZ. Study design: KS, and FY. Data collection: KS, HZ, and WC. Data analysis: KS, BX and XL. Manuscript editing: WC, CF, BT, WL, RG, and WE. Manuscript review: KS and FY. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81801651).

Conflict of interest

Authors CF and WL were employed by Siemens Shenzhen Magnetic Resonance Ltd. Authors BT, RG and WE were employed by Siemens Germany Magnetic Resonance Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.913072/full#supplementary-material

References

1. Lehman CD, Isaacs C, Schnall MD, Pisano ED, Ascher SM, Weatherall PT. Cancer yield of mammography, MR, and US in high-risk women: prospective multi-institution breast cancer screening study. *Radiology* (2007) 244:381–8. doi: 10.1148/radiol.2442060461

2. Mann RM, Cho N, Moy L. Breast MRI: State of the art. *Radiology* (2019) 292:520–36. doi: 10.1148/radiol.2019182947

3. McDonald ES, Romanoff J, Rahbar H, Kitsch AE, Harvey SM, Whisenant JG. Mean apparent diffusion coefficient is a sufficient conventional diffusion-weighted MRI metric to improve breast MRI diagnostic performance: Results from the ECOG-ACRIN cancer research group A6702 diffusion imaging trial. *Radiology* (2020) 298:60–70. doi: 10.1148/radiol.2020202465

4. Marini C, Iacconi C, Giannelli M, Cilotti A, Moretti M, Bartolozzi C. Quantitative diffusion-weighted MR imaging in the differential diagnosis of breast lesion. *Eur Radiol* (2007) 17:2646–55. doi: 10.1007/s00330-007-0621-2

5. Dietzel M, Ellmann S, Schulz-Wendtland R, Clauser P, Wenkel E, Uder M. Breast MRI in the era of diffusion weighted imaging: do we still need signal-intensity time curves? *Eur radiol* (2020) 30:47–56. doi: 10.1007/s00330-019-06346-x

6. Sun K, Chen X, Chai W, Fei X, Fu C, Yan X. Breast cancer: diffusion kurtosis MR imaging–diagnostic accuracy and correlation with clinical-pathologic factors. *Radiology* (2015) 277:46–55. doi: 10.1148/radiol.15141625

7. Bogner W, Pinker-Domenig K, Bickel H, Chmelik M, Weber M, Helbich TH. Readout-segmented echo-planar imaging improves the diagnostic performance of diffusion-weighted MR breast examinations at 3.0 T. *Radiology* (2012) 263:64–76. doi: 10.1148/radiol.12111494

8. Sun K, Chai W, Fu C, Zhan Y, Shen K, Luo X. Diffusion-weighted imaging-guided MR spectroscopy in breast lesions using readout-segmented echo-planar imaging. *Eur Radiol* (2016) 26:1565–74. doi: 10.1007/s00330-015-4000-0

9. Porter DA, Heidemann RM. High resolution diffusion-weighted imaging using readout-segmented echo-planar imaging, parallel imaging and a two-dimensional navigator-based reacquisition. *Magnetic Resonance Med* (2009) 62:468–75. doi: 10.1002/mrm.22024

10. Ohlmeyer S, Laun FB, Palm T, Janka R, Weiland E, Uder M. Simultaneous multislice echo planar imaging for accelerated diffusion-weighted imaging of malignant and benign breast lesions. *Invest Radiol* (2019) 54:524–30. doi: 10.1097/RLI.0000000000000560

11. Setsompop K, Gagoski BA, Polimeni JR, Witzel T, Wedeen VJ, Wald LL. Blipped-controlled aliasing in parallel imaging for simultaneous multislice echo planar imaging with reduced g-factor penalty. *Magnetic Resonance Med* (2012) 67:1210–24. doi: 10.1002/mrm.23097

12. Frost R, Jezzard P, Douaud G, Clare S, Porter DA, Miller KL. Scan time reduction for readout-segmented EPI using simultaneous multislice acceleration: Diffusion-weighted imaging at 3 and 7 Tesla. *Magnetic Resonance Med* (2015) 74:136–49. doi: 10.1002/mrm.25391

13. Song SE, Woo OH, Cho KR, Seo BK, Son YH, Grimm R. Simultaneous multislice readout-segmented echo planar imaging for diffusion-weighted MRI in patients with invasive breast cancers. *J Magnetic Resonance Imaging* (2021) 53:1108–15. doi: 10.1002/jmri.27433

14. Filli L, Ghafoor S, Kenkel D, Liu W, Weiland E, Andreisek G. Simultaneous multi-slice readout-segmented echo planar imaging for accelerated diffusion-weighted imaging of the breast. *Eur J Radiol* (2016) 85:274–8. doi: 10.1016/j.ejrad.2015.10.009

15. Thierfelder KM, Scherr MK, Notohamiprodjo M, Weiß J, Dietrich O, Mueller-Lisse UG. Diffusion-weighted MRI of the prostate: advantages of zoomed EPI with parallel-transmit-accelerated 2D-selective excitation imaging. *Eur Radiol* (2014) 24:3233–41. doi: 10.1007/s00330-014-3347-y

16. Finsterbusch J. Improving the performance of diffusion-weighted inner field-of-view echo-planar imaging based on 2D-selective radiofrequency excitations by tilting the excitation plane. *J Magnetic Resonance Imaging* (2012) 35:984–92. doi: 10.1002/jmri.23522

17. Li C. Diagnostic performance for lymph node staging in head and neck squamous cell carcinoma: Comparison of 18F-FDG PET/CT and syngo ZOOMit echo-planar diffusion-weighted MR imaging. (2014). doi: 10.11588/heidok.00019593

18. Sim KC, Park BJ, Han NY, Sung DJ, Kim MJ, Han YE. Efficacy of ZOOMit coronal diffusion-weighted imaging and MR texture analysis for differentiating between benign and malignant distal bile duct strictures. *Abdominal Radiol* (2020) 45:2418–29. doi: 10.1007/s00261-020-02625-0

19. Sun K, Zhu H, Chai W, Zhan Y, Nickel D, Grimm R. Whole-lesion histogram and texture analyses of breast lesions on inline quantitative DCE mapping with CAIPIRINHA-Dixon-TWIST-VIBE. *Eur Radiol* (2020) 30:57–65. doi: 10.1007/s00330-019-06365-8

20. Xie T, Wang Z, Zhao Q, Bai Q, Zhou X, Gu Y. Machine learning-based analysis of MR multiparametric radiomics for the subtype classification of breast cancer. *Front Oncol* (2019) 9:505. doi: 10.3389/fonc.2019.00505

21. Bougias H, Ghiatas A, Priovolos D, Veliou K, Christou A. Whole-lesion apparent diffusion coefficient (ADC) metrics as a marker of breast tumour characterization–comparison between ADC value and ADC entropy. *Br J Radiol* (2016) 89:20160304. doi: 10.1259/bjr.20160304

22. Kim J-H, Ko ES, Lim Y, Lee KS, Han B-K, Ko EY. Breast cancer heterogeneity: MR imaging texture analysis and survival outcomes. *Radiology* (2017) 282:665–75. doi: 10.1148/radiol.2016160261

23. Fujima N, Homma A, Harada T, Shimizu Y, Tha KK, Kano S. The utility of MRI histogram and texture analysis for the prediction of histological diagnosis in head and neck malignancies. *Cancer Imaging* (2019) 19:1–10. doi: 10.1186/s40644-019-0193-9

24. Rozenberg R, Thornhill RE, Flood TA, Hakim SW, Lim C, Schieda N. Whole-tumor quantitative apparent diffusion coefficient histogram and texture analysis to predict Gleason score upgrading in intermediate-risk 3+ 4= 7 prostate cancer. *Am J Roentgenol* (2016) 206:775–82. doi: 10.2214/AJR.15.15462

25. Xie T, Zhao Q, Fu C, Bai Q, Zhou X, Li L. Differentiation of triple-negative breast cancer from other subtypes through whole-tumor histogram analysis on multiparametric MR imaging. *Eur Radiol* (2019) 29:2535–44. doi: 10.1007/s00330-018-5804-5

26. Jiang J-S, Zhu L-N, Wu Q, Sun Y, Liu W, Xu X-Q. Feasibility study of using simultaneous multi-slice RESOLVE diffusion weighted imaging to assess

parotid gland tumors: comparison with conventional RESOLVE diffusion weighted imaging. *BMC Med Imaging* (2020) 20:1–8. doi: 10.1186/s12880-020-00492-1

27. Hu Y, Zhan C, Yang Z, Zhang X, Zhang H, Liu W. Accelerating acquisition of readout-segmented echo planar imaging with a simultaneous multi-slice (SMS) technique for diagnosing breast lesions. *Eur Radiol* (2021) 31:2667–76. doi: 10.1007/s00330-020-07393-5

28. Suo S, Zhang D, Cheng F, Cao M, Hua J, Lu J. Added value of mean and entropy of apparent diffusion coefficient values for evaluating histologic phenotypes of invasive ductal breast cancer with MR imaging. *Eur Radiol* (2019) 29:1425–34. doi: 10.1007/s00330-018-5667-9

29. Rivenbark AG, O'Connor SM, Coleman WB. Molecular and cellular heterogeneity in breast cancer: challenges for personalized medicine. *Am J Pathol* (2013) 183:1113–24. doi: 10.1016/j.ajpath.2013.08.002

30. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature* (2013) 501:328–37. doi: 10.1038/nature12624

31. Gnep K, Fargeas A, Gutiérrez-Carvajal RE, Commandeur F, Mathieu R, Ospina JD. Haralick textural features on T2-weighted MRI are associated with biochemical recurrence following radiotherapy for peripheral zone prostate cancer. *J Magnetic Resonance Imaging* (2017) 45:103–17. doi: 10.1002/jmri.25335

32. Reeder SB, Wintersperger BJ, Dietrich O, Lanz T, Greiser A, Reiser MF. Practical approaches to the evaluation of signal-to-noise ratio performance with parallel imaging: application with cardiac imaging and a 32-channel cardiac coil. *Magnetic Resonance Med* (2005) 54:748–54. doi: 10.1002/mrm.20636

33. Park JY, Shin HJ, Shin KC, Sung YS, Choi WJ, Chae EY. Comparison of readout segmented echo planar imaging (EPI) and EPI with reduced field-of-view diffusion-weighted imaging at 3T in patients with breast cancer. *J Magnetic Resonance Imaging* (2015) 42:1679–88. doi: 10.1002/jmri.24940

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 26 August 2022
DOI 10.3389/fonc.2022.918757

OPEN ACCESS

EDITED BY
Ariella Hanker,
University of Texas Southwestern Medical Center, United States

REVIEWED BY
Coy D. Heldermon,
University of Florida, United States
Ed Esplin,
Invitae, United States

*CORRESPONDENCE
Rachel E. Ellsworth
rachel.ellsworth.ctr@usuhs.edu

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 12 April 2022
ACCEPTED 04 August 2022
PUBLISHED 26 August 2022

CITATION
Turza L, Lovejoy LA, Turner CE, Shriver CD and Ellsworth RE (2022) Eligibility, uptake and response to germline genetic testing in women with DCIS.
Front. Oncol. 12:918757.
doi: 10.3389/fonc.2022.918757

Eligibility, uptake and response to germline genetic testing in women with DCIS

Lauren Turza[1], Leann A. Lovejoy[2], Clesson E. Turner[3,4], Craig D. Shriver[3] and Rachel E. Ellsworth[3,5]*

[1]Department of Surgery, Rebecca Fortney Breast Center, Anne Arundel Medical Center, Annapolis, MD, United States, [2]Clinical Breast Care Project, Chan Soon-Shiong Institute of Molecular Medicine at Windber, Windber, PA, United States, [3]Murtha Cancer Center Research Program, Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [4]National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, United States, [5]Henry M. Jackson Foundation for the Advancement of Military Medicine, Bethesda, MD, United States

Background: Ductal carcinoma *in situ* (DCIS) is a malignant, yet pre-invasive disease of the breast. While the majority of DCIS have low risk of recurrence, a subset of women with germline pathogenic variants (PV) in cancer predisposition genes are at increased risk for recurrence. Uptake of genetic testing and subsequent surgical intervention in women with DCIS has not been well-studied. The aim of this study was to evaluate test eligibility parameters, uptake of clinical testing, impact on surgical decision making and second cancer events (SCE) in women with DCIS.

Methods: Four-hundred eighty-four women diagnosed with unilateral DCIS 2001-2020 were eligible for this study. Demographic, commercial genetic test results and surgical procedures were extracted from the database. Test-eligibility was assigned using National Comprehensive Cancer Network (NCCN) criteria. Panel genetic testing was performed in the research laboratory across 94 cancer predisposition genes. Statistical analyses were performed using Fisher's exact tests and Chi-square analyses with $p < 0.05$ defining significance.

Results: Forty-four percent of women were test-eligible at diagnosis of which 63.4% pursued genetic testing before definitive surgery; 9.9% pursued testing only after a second cancer event. Bilateral mastectomy (BM) was significantly higher (p<0.001) in women who had testing before definitive surgery (46.9%) compared to those who had testing afterword (10.8%) and in women who underwent testing before definitive surgery with PV (75%) compared to those without PV (37.5%. p=0.045). Of the 39 women with PV, 20 (51.3%) were detected only in the research setting, with 7 (17.9%) of these women not eligible for genetic testing based on NCCN criteria. In women who did not undergo BM at diagnosis, SCE were significantly higher (p=0.001) in women with PV (33.3%) compared to those without PV (11.9%).

Conclusion: Pursuit of genetic testing and subsequent use of risk-reducing surgeries in women with PV was suboptimal in women with a primary diagnosis

10.3389/fonc.2022.918757

of DCIS. In conjunction, >50% of PV were detected only in the research setting. Because omission of genetic testing in women with DCIS may represent a lost opportunity for prevention, genetic testing at the time of diagnosis should be standard for all women with DCIS.

KEYWORDS

ductal carcinoma *in situ*, genetic testing, germline mutation, risk-reducing surgery, recurrence

Introduction

Ductal carcinoma *in situ* (DCIS) is a disease of the breast in which epithelial cellular proliferation fills terminal ductal lobular units with malignant cells. Although a pre-invasive condition, DCIS is a non-obligate precursor to invasive breast cancer (IBC) (1). Studies of the natural progression of DCIS found that 14-53% of untreated lesions progressed to IBC (2). Current treatment options for DCIS include surgery, radiation and endocrine therapy (1).

The primary goal of treatment of DCIS is to prevent recurrence (3). To avoid over- or under-treating indolent or aggressive DCIS, a number of studies and clinical trials are attempting to identify pathological characteristics and biomarkers associated with risk of recurrence and evaluating whether active surveillance is an acceptable alternative to surgery for some women (4). One subset of women with DCIS who may benefit not only from more extensive breast surgical options including bilateral mastectomy (BM), but bilateral salpingo-oophorectomy as well, are those who harbor pathogenic variants (PV) in cancer predisposition genes associated with increased risk for recurrence and secondary cancers at other sites.

In the United States, guidelines, such as those issued by the National Comprehensive Cancer Network (NCCN), are used to identify those women with breast cancer, both invasive and DCIS, who are likely to benefit from germline genetic testing. Recent studies suggest that a significant number of women with PV do not meet testing criteria (5–7) and thus miss the opportunity to pursue risk-reducing strategies, including BM, at diagnosis. In response, the American Society of Breast Surgeons (ASBS) recommends genetic testing should be offered to all women with breast cancer (8). This study was designed to evaluate how effective current test eligibility parameters are in identifying women with germline mutations in cancer predisposition genes, uptake and timing of genetic testing, choice of risk-reducing surgery in those with PV and second cancer events (SCE) in a cohort of 484 women with a primary diagnosis of unilateral DCIS.

Materials and methods

Patient eligibility and enrollment

All subjects in this study voluntarily agreed to participate in the Clinical Breast Care Project (CBCP). Patients were enrolled and treated at the CBCP member sites of Walter Reed National Military Medical Center (WRNMMC), Bethesda, MD (n=266), Anne Arundel Medical Center, Annapolis, MD (n=159) or Joyce Murtha Breast Care Center, Windber, PA (n=59). Demographic and clinical data and blood samples were collected with approval from the WRNMMC Human Use Committee and Institutional Review Board.

Patient data

Demographic, personal and family health history and lifestyle factors were collected and entered into the CBCP database at the time of diagnosis. Clinical data such as pathological characteristics and germline test results were entered into the CBCP database as they became available. Follow-up data, including any additional cancer events, was collected annually and entered into the CBCP database. Eligibility for germline testing was assigned using National Comprehensive Cancer Network (NCCN) criteria, which includes age at diagnosis and personal and family cancer history, both from the year of diagnosis and criteria from 2021 (version 2.2021). Type of surgical management, local, regional and distant SCE and patient status through December 31, 2021 were collected. Results from clinical genetic testing (n=110) were extracted from the CBCP database.

Data generation and analysis

Genomic DNA was available from 465 women, 92 of whom also had clinical genetic testing performed. Genomic DNA was

isolated from blood samples as previously described (9). Sequencing libraries were created using Illumina DNA prep with enrichment kits and the TruSight Cancer panel and sequenced on a MiSeq (Illumina, Inc, San Diego, CA) according to manufacturer's protocols. Data were analyzed and variants classified as previously described (9). Statistical analyses were performed using Fisher's exact tests and Chi-square analyses. Statistical significance was defined as $p < 0.05$.

Results

Cohort characteristics

Four hundred eighty-four women with a primary diagnosis of unilateral DCIS were eligible for this study. The average age at diagnosis in this cohort was 57 years. Average follow-up time was 8.6 years. Fifty-nine (12.2%) women had second cancer events including 13 recurrences (6 ipsilateral DCIS, 7 ipsilateral IBC) and 43 second cancer events (7 DCIS, 36 IBC), ovarian cancer (n=1) and metastatic spread without detection of IBC (n=2). One woman who developed ipsilateral invasive breast cancer died of disease.

Test eligibility

Two-hundred fourteen women met at least one NCCN criteria for genetic testing at the time of diagnosis (Figure 1). An additional 66 women met test criteria from 2021, including 21 women whose test status changed only after subsequent diagnosis of recurrent DCIS (n=3), IBC (n=17) or ovarian cancer (n=1). There was no significant difference in patient self-reported race/ethnicity between risk-groups, however, women who were test-eligible at the time of diagnosis had more relatives with breast, ovarian or pancreatic than those with delayed test-eligibility and second cancer events were lowest in low-risk patients (Table 1).

Test uptake

Overall, 39.3% (110/280) of test eligible women underwent clinical genetic testing. Test uptake was significantly higher (p<0.001) in women who were eligible at the time of testing (47.2%) compared to those with delayed eligibility (13.6%). In women who were test-eligible at the time of diagnosis, those that underwent genetic testing were significantly younger than those who did not pursue genetic testing and were more likely to have ≥3 family members with a history of cancer (Table 2).

Timing of test uptake

Timing of genetic testing varied. Time-to-testing was significantly (p<0.001) longer in women who became eligible after diagnosis (average 8.94 years, range 3.1 -14.9 years) than those who were eligible at the time of diagnosis (average 2.15 years, range 0-18.5 years). Within the test-eligible at diagnosis cohort of women, 63.4% (64/101) pursued genetic testing before definitive surgery. Ten (9.9%) of the women eligible for testing at diagnosis delayed testing until after a second cancer event. Each of the nine women with delayed eligibility for testing pursued clinical genetic testing after a second cancer event.

Overall mutation rates

The mutation frequency of women with clinical test results was 17.3% (19/110). Two women who had clinical testing limited to *BRCA1* and *BRCA2*, had PV in *ATM* (n=1) and *CHEK2* (n=1) detected in the research setting. Eleven (6.5%) of an additional 170 test-eligible women who did not pursue clinical testing had PV detected in the research laboratory. Within the test-ineligible population, 7/204 (3.4%) women had PV. In total, 20/39 (51.3%) PV were detected in women who did not undergo clinical genetic testing. PV were detected in 13 cancer predisposition genes including *ATM* (n=4), *BLM* (n=1), *BRCA1* (n=1), *BRCA2* (n=7), *BRIP1* (n=1), *CDKN2A* (n=1), *CHEK2* (n=9), *FANCC* (n=1), *MUTYH* (n=10), *NBN* (n=1), *PALB2* (n=1), *RAD51D* (n=1) and *STK11* (n=1) (Table 3). *CHEK2* (n=9) and *BRCA2* (n=7) had the highest frequency of PV in high-risk women while *MUTYH* had the most PV in low-risk women.

Surgical choices and outcomes

Within the 101 women who underwent clinical genetic testing, BM was significantly higher (p<0.001) in women who had testing before definitive surgery (46.9%) compared to those who had testing after definitive surgery (10.8%). Within the women with clinically-detected PV, 36.8% elected for BM. In women who underwent testing before definitive surgery, BM was significantly higher (p=0.045) in those with PV (75%) compared to those without (42.9%). In women who received negative test results before definitive surgery, the rate of SCE was 0% in women who underwent BM and 6% in those who did not (p=0.212). The number of PV in high- and low-risk women who had SCE is shown in Table 4. In women who did not undergo BM at the time of diagnosis, SCE were significantly higher (p=0.001) in women with PV (10/30; 33.3%) compared to those women without PV (46/388, 11.9%). None of the women with PV died of disease.

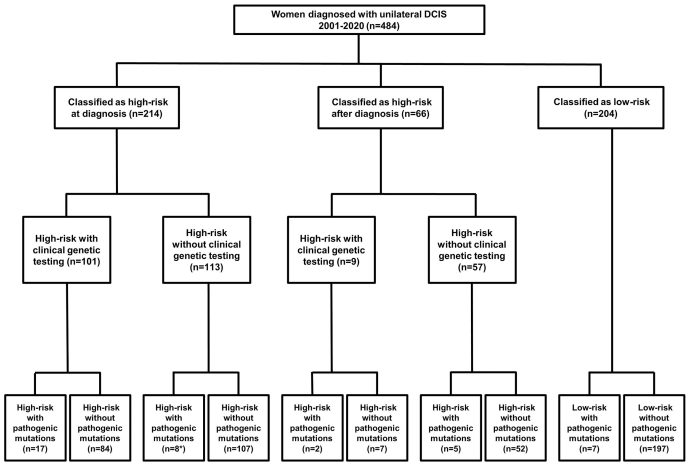


FIGURE 1
Flow-chart detailing patient risk, test uptake and detection of pathogenic mutations. *Two women who had clinical testing limited to *BRCA1* and *BRCA2* with no PV reported had PV in the *ATM* and *CHEK2* genes detected in the research setting.

Discussion

The primary goal of treatment for DCIS is prevention of IBC. Although a significant number of women have indolent forms of DCIS and may be effectively treated using active surveillance rather than surgical interventions (4), those with hereditary forms of DCIS are at increased risk for additional breast cancers, both ipsilateral and contralateral, as well as secondary tumors in other organ sites. For these patients, surgical management may be more extensive and include BM for women with PV in *BRCA1*, *BRCA2*, *PALB2* and *TP53*, and BSO for women with PV in *BRCA1*, *BRCA2, BRIP1*, *RAD51C*

TABLE 1 Demographic and clinical information for all patients classified as high-risk at the time of diagnosis, high-risk after diagnosis or low-risk using NCCN criteria.

	High-risk at diagnosis (n=214)	High-risk after diagnosis (n=66)	p-value[a]	Low-risk (n=204)	p-value[b]
Age at Diagnosis	52.3 years	53.0 years	0.680	62.8 years	<0.001
	N (%)	N (%)		N (%)	
Ethnicity			0.879		0.417
Non-Hispanic Black	46 (21.5%)	12 (18.2%)		34 (16.7%)	
Asian/Pacific Islander	7 (3.3%)	3 (4.5%)		12 (5.9%)	
Hispanic	7 (3.3%)	2 (3.0%)		6 (2.9%)	
Non-Hispanic White	152 (71.0%)	49 (74.3%)		148 (72.5%)	
Other/Unknown	2 (0.9%)	0 (0.0%)		4 (2.0%)	
Family history[c]			<0.001		<0.001
0	41[d] (19.2%)	16 (24.2%)		139 (68.1%)	
1	53 (24.9%)	46 (69.7%)		65 (31.9%)	
2	75 (35.2%)	3 (4.6%)		0 (0.0%)	
>3	44 (20.7%)	1 (1.5%)		0 (0.0%)	
Disease-recurrence			<0.001		<0.001
Yes	28 (13.1%)	21 (31.8%)		10 (4.9%)	
No	186 (86.9%)	45 (68.2%)		194 (95.1%)	

[a]p-value for women who were test eligible at compared to after diagnosis.
[b]p-value for high-risk compared to low-risk women.
[c]Family history of breast, ovarian and pancreatic cancer through third degree family members.

TABLE 2 Demographic and clinical information for patients classified as test eligible at the time of diagnosis who did or did not pursue genetic testing.

	High-risk tested (n=101)	High-risk not tested (n=113)	P-value
Age at Diagnosis	49.1 years	55.1 years	0.002
	N (%)	N (%)	
Ethnicity			0.455
Non-Hispanic Black	25 (24.7%)	21 (18.6%)	
Asian/Pacific Islander	4 (4.0%)	3 (2.7%)	
Hispanic	4 (4.0%)	3 (2.7%)	
Non-Hispanic White	68 (67.3%)	84 (74.3%)	
Other/Unknown	0 (0.0%)	2 (1.7%)	
Education			0.146
<college degree	38 (37.6%)	55 (48.7%)	
≥college degree	59 (58.4%)	51 (45.1%)	
Unknown	4 (4.0%)	7 (6.2%)	
Family History			0.028
0	22 (21.8%)	20 (17.7%)	
1	24 (23.8%)	29 (25.7%)	
2	27 (26.7%)	48 (42.5%)	
≥3	28 (27.7%)	16 (14.1%)	

and *RAD51D* (10, 11). Identification of women with heritable forms of DCIS is, therefore, critical in surgical decision making and preventing disease recurrence.

Efficacy of germline testing in reducing the risk of additional cancers in patients with DCIS is dependent on several factors, including test-eligibility, timing of genetic testing and treatment decisions based on underlying PV. In this study, 44.2% of women were eligible for testing at diagnosis; this was not significantly different (p=0.944) from a cohort of women with IBC (44.0%) diagnosed over the same time period (9); test uptake was <50% in test-eligible at diagnosis women with DCIS (47.2%) but not significantly lower (p=0.241) than those with IBC (51.8%). In our study, 40.6% (13 of 32) of PV in test-eligible women were not detected clinically. In addition, the frequency of PV in test-ineligible women was 3.4% in women with DCIS, similar to the 4.0% detected in women with invasive breast cancer (9). These data suggest that the use of test-eligibility criteria may create a failed opportunity for prevention; implementation of the the ASBS recommendation that all women with breast cancer should be offered genetic testing (8) may reduce risk of recurrence in women with DCIS.

The timing of genetic testing also is also important as it can lead to changes in surgical decision making. Of the 101 women who were test-eligible at the time of diagnosis with clinical test results, 36 (35.6%) underwent genetic testing after definitive surgery for DCIS, with 10 (9.9%) delaying testing until after a second cancer event. Ten (27.0%) women with delayed testing were found to harbor PV, six of whom had PV in genes for which NCCN recommendations for patient management are available.

Genetic testing results can reduce future cancer risk when women utilize test results to guide treatment. For example, NCCN guidelines suggest risk-reducing mastectomy and BSO should be considered for women with *BRCA1* and *BRCA2* mutations (12). In our study, of the six women with clinically-detected PV in *BRCA2*, three underwent BM and BSO at the time of diagnosis and remain cancer free. The remaining three women with PVs in *BRCA2* developed ipsilateral breast cancer with one woman having ovarian cancer found incidentally during her subsequent BSO. Each of these three women was test-eligible at the time of DCIS diagnosis and could have potentially prevented additional cancers had they pursued timely genetic testing and risk-reducing surgeries.

While the benefit of risk-reducing surgeries in preventing second cancers in women with DCIS and PV in *BRCA1* or *BRCA2* are well established, preventing recurrence without overtreatment for women with PV in other genes is more challenging. This is of considerable importance as within our cohort, 31/39 PV were in genes other than *BRCA1* and *BRCA2*. Of note, nine (1.9%) of women had PV in *CHEK2* of which two had second cancer events. Currently, enhanced surveillance rather than risk-reducing surgery is recommended for women with *CHEK2* PV (12). In conjunction with our results, Petridis et al. found that 5/16 (31%) of women with DCIS and *CHEK2* mutations developed contralateral disease (13) and a recent study of germline variants in patients with second breast cancers found that *CHEK2* was the most frequently mutated gene in women with second breast cancers (3.4%) (14). Thus, the risk of recurrence for women with DCIS and *CHEK2* PV may warrant the use of risk-reducing surgery.

10.3389/fonc.2022.918757

TABLE 3 Variants classified as pathogenic or likely pathogenic according to ACMG classification.

Patient	Pathogenic variant	Testing before definitive surgery	Surgery at diagnosis[a]	Recurrence
Test-eligible with clinical testing				
17	BRCA2: exact mutation from clinical lab not provided	√	BM/BSO	
48[b]	NM_007194.4(CHEK2):c.470T>C (p.Ile157Thr)		UM	Contralateral IBC[c]
68	NM_007194.4(CHEK2):c.906delA (p.Glu302fs)		BM	
156	NM_000077.4(CDKN2A):c.301G>T (p.Gly101Trp)		BCS	
188	NM_024675.4(PALB2):c.509_510delGA (p.Arg170fs)	√	BM/BSO	
190	NM_001048174.2(MUTYH):c.1103G>A (p.Gly368Asp)		BCS	
364	NM_012222.2(MUTYH):c.724C>T (p.Arg242Cys)		BCS	
369	NM_007194.4(CHEK2):c.85C>T (p.Gln29Ter)		BCS/BSO	
379	NM_000059.4(BRCA2):c.518delG (p.Gly173fs)	√	BM/BSO	
416	NM_000059.4(BRCA2):c.3975_3978dupTGCT (p.Ala1327fs)	√	BCS	Ipsilateral IBC
429	NM_000059.3(BRCA2):c.8902_8913delACCGTGTGGAAinsTCCC (p.Thr2968fs)	√	BM/BSO	
431	NM_000059.4(BRCA2):c.5946delT (p.Ser1982fs)	√	BCS/BSO	Ipsilateral IBC
445	NM_032043.2(BRIP1):c.1045G>C (p.Ala349Pro)		BCS	Ipsilateral IBC
468	STK11: partial gene deletion	√	BM	
476	CHEK2: deletion exons 9-10	√	BM	
504 [b]	NM_000051.4(ATM):c.6706G>T (p.Glu2236*)		BCS	Ipsilateral IBC
505	NM_000059.4(BRCA2):c.1310_1313del (p.Lys437fs)		BCS	Ipsilateral IBC and OC[d]
518	NM_001048174.2(MUTYH):c.1103G>A (p.Gly368Asp)		BCS	Ipsilateral DCIS
559	NM_007194.4(CHEK2):c.470T>C (p.Ile157Thr)		BCS	
Test-eligible with research results				
82	NM_000057.4(BLM):c.1933C>T (p.Gln645*)		BCS	
97	NM_000059.3(BRCA2):c.2842dupG (p.Val948fs)		UM	Contralateral IBC
120	NM_002485.5(NBN):c.698_701del (p.Lys233fs)		BM	
143[e]	NM_000051.4(ATM):c.6228del (p.Leu2077fs)		BCS	
230	NM_002878.3(RAD51D):c.694C>T (p.Arg232*)		BM	
240[e]	NM_007194.4(CHEK2):c.1100delC (p.Thr367fs)		BCS	Ipsilateral DCIS
276	NM_007194.4(CHEK2):c.1100delC (p.Thr367fs)		BCS	
293	NM_007194.4(CHEK2):c.349A>G (p.Arg117Gly)		BCS	
297	NM_000136.3(FANCC):c.355_360delinsA (p.Ser119fs)		UM	
319	NM_007194.4(CHEK2):c.1100delC (p.Thr367fs)		BCS	
405	NM_001048174.2(MUTYH):c.1351G>T (p.Glu451Ter)		BCS	Ipsilateral IBC
427	NM_012222.2(MUTYH):c.724C>T (p.Arg242Cys)		BCS	
511	NM_007294.4(BRCA1):c.4035delA (p.Glu1346fs)		BCS	
Test-ineligible with research results				
181	NM_001048174.2(MUTYH):c.452A>G (p.Tyr151Cys)		BCS	
231	NM_000051.3(ATM):c.7096G>T (p.Glu2366*)		BCS	
237	NM_001048174.2(MUTYH):c.452A>G (p.Tyr151Cys)		BCS	
339	NM_000051.4(ATM):c.1564_1565delGA (p.Glu522fs)		BCS	
388	NM_001048174.2(MUTYH):c.1103G>A (p.Gly368Asp)		BCS	
423	NM_001048174.2(MUTYH):c.1103G>A (p.Gly368Asp)		UM	
469	NM_001048174.2(MUTYH):c.1103G>A (p.Gly368Asp)		BCS	

[a]BCS, breast conserving surgery; UM, unilateral mastectomy; BM, bilateral mastectomy; BSO, bilateral salpingo oophorectomy.
[b]Patients became eligible for testing only after development of a second breast tumor.
[c]IBC, invasive breast cancer.
[d]OC, ovarian cancer.
[e]Patients had genetic testing of BRCA1 and BRCA2 done clinically; ATM and CHEK2 mutation detected in the research setting.

TABLE 4 Risk-status and germline variants in 59 women with second cancer events.

	Ipsilateral DCIS	Contralateral DCIS	Ipsilateral Invasive	Contralateral Invasive	Ovary	Metastatic with no IBC[a]
High-Risk[b]						
High-penetrance	0 (0%)	0 (0%)	[c]3 (12.5%)	1 (8.3%)	0 (0%)	0 (0%)
Moderate-penetrance	[d]1 (14.3%)	0 (0%)	2 (8.3%)	1 (8.3%)	0 (0%)	0 (0%)
Other	[e]1 (14.3%)	0 (0%)	1 (4.2%)	0 (0%)	0 (0%)	0 (0%)
No PV	[f]5 (71.4%)	3 (100%)	[h]18 (75.0%)	10 (83.3%)	1 (100%)	2 (100%)
Low-Risk						
High-penetrance	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Moderate-penetrance	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
No PV	2[g] (100%)	1 (100%)	3 [i] (100%)	4 (100%)	0 (0%)	0 (0%)

[a]IBC, invasive breast cancer.
[b]High-penetrance genes: BRCA2, moderate penetrance genes: ATM, BRIP1, CHEK2.
[c]1 of 3 patients had recurrent disease.
[d]This patient had recurrent disease.
[e]This patient had recurrent disease.
[f]2 of 5 patients had recurrent disease.
[g]5 of 18 patients had recurrent disease.
[h]1 of 2 patients had recurrent disease.
[i]2 of 3 patients had recurrent disease.

In conjunction with the high frequency of PV in *CHEK2*, *MUTYH* (n=10, 2.1%) was the gene with the highest frequency of PV in our study, with two of the 10 women recurring. While a study of over 30,000 women with breast cancer found no significant increase in risk of breast cancer in women with *MUTYH* (15), a recent study of 165 women with *BRCA1/2*-negative IBC found that *MUTYH* was the most commonly mutated gene (3.6%) (16). The use of expanded germline panel testing may add complexity to patients understanding of DCIS and surgical decision making.

There are several limitations to this study. Data were not available for pre- or post-test counseling, thus, it is not possible to determine how many patients did not undergo genetic testing because they were not offered the opportunity and how many declined testing. In addition, the reasons behind surgical decision making were not collected, thus, we were unable to determine why in women who had testing before definitive surgery, 42.9% of women who received negative test results elected to undergo BM and 40% with a *BRCA2* PV chose BCS. Rates of contralateral breast cancer are low in women undergoing BCS, with use of radiation associated with lower risk for ipsilateral breast cancer and endocrine therapy associated with risk of contralateral breast cancer (17). BM may, therefore, represent overtreatment for the majority of patients with DCIS. Genetic testing to identify those women with PV at increased risk for SCE provides important information that will allow the patient to develop an individualized and tailored breast cancer treatment plan. Surgical decision should balance the risks and morbidity associated with BM with desired cosmesis, extent of DCIS, concerns about side effects of radiation therapy and fear of recurrence. Finally, although germline status was available for 484 women in this study, the number of women with PV was small. Thus, care must be taken in interpreting risk of SCE or using these data to influence surgical decision making.

In conclusion, 8.1% of women with unilateral DCIS had detectable germline mutations, including 3.4% of women not currently eligible for genetic testing. Less than half of the eligible women pursued genetic testing, and 10% did so only after a second cancer event. Half of the women with PV in genes for which prophylactic surgery should be discussed did not undergo BM and recurred. Given that more than 50% of PV were detected only in the research setting and that SCE were significantly higher in women with PV compared to those without, we suggest that, in accordance with recommendations from the ASBS, germline testing should be offered to all women diagnosed with DCIS, and in fact, should be included as standard-of-care at the time of diagnosis. Future studies to identify the factors associated with the suboptimal pursuit of genetic testing and subsequent risk-reducing surgeries are critical to reduce the risk of second cancer events in women with DCIS.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Walter Reed National Military Medical Center Human Use Committee and Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LT reviewed the data and provided extensive revisions to the manuscript; LL generated panel test sequencing data and contributed to revisions of the manuscript; CT provided clinical interpretation of the data and revision of the manuscript, CS provided resources for the study and revision of the manuscript, RE conceived of the project, performed data analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by a cooperative agreement from the Uniformed Services University of the Health Sciences HU0001-16-2-0004 through the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.

Acknowledgments

The contents of this publication are the sole responsibility of the author(s) and do not necessarily reflect the views, opinions or policies of the Uniformed Services University of the Health Sciences (USUHS), the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., the Department of Defense (DoD) or the Departments of the Army, Navy, or Air Force. Mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. Government.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Tomlinson-Hansen S, Khan M, Cassaro S. Breast ductal carcinoma *in situ*. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing (2021)

2. Erbas B, Provenzano E, Armes J, Gertig D. The natural history of ductal carcinoma *in situ* of the breast: A review. *Breast Cancer Res Treat* (2006) 97:135–44. doi: 10.1007/s10549-005-9101-z

3. American Cancer Society. *Cancer facts and figures 2015: Special section: breast carcinoma in situ*. Atlanta: American Cancer Society (2015).

4. Groen EJ, Elshof LE, Visser LL, Rutgers EJT, Winter-Warnars HAO, Lips EH, et al. Finding the balance between over- and under-treatment of ductal carcinoma *in situ* (DCIS). *Breast* (2017) 31:274–83. doi: 10.1016/j.breast.2016.09.001

5. Beitsch PD, Whitworth PW, Hughes K, Patel R, Rosen B, Compagnoni G, et al. Underdiagnosis of hereditary breast cancer: Are genetic testing guidelines a tool or an obstacle? *J Clin Oncol* (2019) 37:453–60. doi: 10.1200/JCO.18.01631

6. Samadder NJ, Riegert-Johnson D, Boardman L, Rhodes D, Wick M, Okuno S, et al. Comparison of universal genetic testing vs guideline-directed targeted testing for patients with hereditary cancer syndrome. *JAMA Oncol* (2021) 7:230–7. doi: 10.1001/jamaoncol.2020.6252

7. Yang S, Axilbund JE, O'Leary E, Michalski ST, Evans R, Lincoln SE, et al. Underdiagnosis of hereditary breast and ovarian cancer in Medicare patients: Genetic testing criteria miss the mark. *Ann Surg Oncol* (2018) 25:2925–31. doi: 10.1245/s10434-018-6621-4

8. Manahan ER, Kuerer HM, Sebastian M, Hughes KS, Boughey JC, Euhus DM, et al. Consensus guidelines on genetic` testing for hereditary breast cancer from the American society of breast surgeons. *Ann Surg Oncol* (2019) 26:3025–31. doi: 10.1245/s10434-019-07549-8

9. Rummel SK, Lovejoy LA, Turner CE, Shriver CD, Ellsworth RE. Should genetic testing for cancer predisposition be standard-of-Care for women with invasive breast cancer? The murtha cancer center experience. *Cancers (Basel)* (2020) 12:234. doi: 10.3390/cancers12010234

10. National Comprehensive Cancer Network. *NCCN clinical practice guidelines in oncology (NCCN guidelines): Breast cancer*. Plymouth Meeting, PA: National Comprehensive Cancer Network, Inc. (2021).

11. Tung NM, Boughey JC, Pierce LJ, Robson ME, Bedrosian I, Dietz JR, et al. Management of hereditary breast cancer: American society of clinical oncology, American society for radiation oncology, and society of surgical oncology guideline. *J Clin Oncol* (2020) 38:2080–106. doi: 10.1200/JCO.20.00299

12. National Comprehensive Cancer Network. *Genetic/familial high-risk assessment: Breast, ovarian, and pancreatic cancer*. Plymouth Meeting, PA: National Comprehensive Cancer Network, Inc. (2021) National Comprehensive Cancer Network, Inc. Plymouth Meeting, PA.

13. Petridis C, Arora I, Shah V, Megalios A, Moss C, Mera A, et al. Frequency of pathogenic germline variants in BRCA1, BRCA2, PALB2, CHEK2 and TP53 in ductal carcinoma *in situ* diagnosed in women under the age of 50 years. *Breast Cancer Res* (2019) 21:58. doi: 10.1186/s13058-019-1143-y

14. Yao KK, Clifford J, Li S, LaDuca H, Hulick P, Gutierrez S, et al. Prevalence of germline pathogenic and likely pathogenic variants in patients with second breast cancers. *JNCI Cancer Spectr* (2020) 4:pkaa094. doi: 10.1093/jncics/pkaa094

15. Fulk K, LaDuca H, Black MH, Qian D, Tian Y, Yussuf A, et al. Monoallelic MUTYH carrier status is not associated with increased breast cancer risk in a multigene panel cohort. *Fam Cancer* (2019) 18:197–201. doi: 10.1007/s10689-018-00114-4

16. Bono M, Fanale D, Incorvaia L, Cancelliere D, Fiorino A, Calo V, et al. Impact of deleterious variants in other genes beyond BRCA1/2 detected in breast/ovarian and pancreatic cancer patients by NGS-based multi-gene panel testing: Looking over the hedge. *ESMO Open* (2021) 6:100235. doi: 10.1016/j.esmoop.2021.100235

17. Miller ME, Muhsen S, Olcese C, Patil S, Morrow M, Van Zee KJ. Contralateral breast cancer risk in women with ductal carcinoma in situ: Is it high enough to justify bilateral mastectomy? *Ann Surg Oncol* (2017) 24:2889–97. doi: 10.1245/s10434-017-5931-2

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 29 August 2022
DOI 10.3389/fonc.2022.920211

OPEN ACCESS

EDITED BY
San-Gang Wu,
First Affiliated Hospital of Xiamen University, China

REVIEWED BY
Christin A. Knowlton,
Yale University, United States
Assie Olfatbakhsh,
Motamed Cancer Institute, Iran

*CORRESPONDENCE
Andrzej Szopa
aszopa@sum.edu.pl

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 14 April 2022
ACCEPTED 10 August 2022
PUBLISHED 29 August 2022

CITATION
Koralewska A, Domagalska-Szopa M, Łukowski R and Szopa A (2022) Influence of the external breast prosthesis on the postural control of women who underwent mastectomy: Cross-sectional study.
Front. Oncol. 12:920211.
doi: 10.3389/fonc.2022.920211

Influence of the external breast prosthesis on the postural control of women who underwent mastectomy: Cross-sectional study

Anna Koralewska[1], Małgorzata Domagalska-Szopa[1], Robert Łukowski[2] and Andrzej Szopa[3,4]*

[1]Department of Developmental Age Physiotherapy, Medical University of Silesia, Katowice, Poland, [2]Medical Clinic Sanus, Zabrze, Poland, [3]Department of Physiotherapy, Medical University of Silesia, Katowice, Poland, [4]Neuromed, Rehabilitation and Medical Center, Katowice, Poland

Background: Women after mastectomy may decide to either have a breast reconstruction or use an external breast prosthesis. Aim: The aim of the presented research was to evaluate the influence of external breast prosthesis on postural stability in women after mastectomy. Methods and Procedures: In the study 52 women after unilateral mastectomy took part. The study consisted of 4 parts: 1) anthropometric measurements; 2) measurements of upper limb circumference; 3) assessment of weight-bearing (WB); and 4) posturographic tests (PT). Outcomes and Results: Differences in the arm circumferences on the amputated (A) and non-amputated (NA) sides did not confirm the occurrence of lymphedema in limb on amputated side. The results of the WB between the A and NA body sides in both tested conditions, i.e., with open and closed eyes, showed no significant differences between the test with and without an external prosthesis. No statistically differences have been observed between posturometric parameters with and without breast prosthesis during both PT. In comparing the posturometric parameters between the PT with open and closed eyes, the sway path of the center of pressure was statistically significantly longer when eyes were closed in both conditions, i.e., with and without breast prosthesis. Conclusion and Implications: The finding show that 1) external breast prosthesis does not have a significant influence on the symmetry of loading on the A and NA body sides and on the postural stability of women after unilateral mastectomy and 2) exclusion visual control during PT increases postural instability in women after unilateral mastectomy.

KEYWORDS

mastectomy, external breast prosthesis, postural control, stabilographic platform, posturography

1 Introduction

Breast cancer is considered the greatest oncological problem in developed countries and a constantly growing problem in developing countries (1–4). According to the National Cancer Registry, malignant neoplasms are the second most common cause of death in Poland, while breast cancer is the second most common cancer-related cause of death in women (15%) (5). Considering the predicted demographic changes in the Polish population and the fact that the highest percentage of cases is noted in individuals aged >50 years, it can be expected that by 2025, >80,000 women in Poland will develop breast cancer (6).

There are several well-established cancer treatment procedures such as radiotherapy, hormone therapy, chemotherapy, biological treatment, and surgical treatment.

The reduced invasiveness of breast cancer surgical treatment is a widely acknowledged preference; hence, there is a tendency to perform the breast-conserving procedure, which increase the quality of patient's life (7, 8). Nevertheless, sometimes it is necessary surgical treatment to remove the entire tumor along with the entire mammary gland, the fascia of the pectoralis major, and the lymph nodes (Modified Radical Mastectomy or Simple Mastectomy), which is always associated with a structural and functional deficit (2, 9, 10).

A large proportion of women worldwide decide to undergo breast reconstruction after mastectomy. However, in Poland, only 20%–40% of women decide to undergo this type of surgery after breast removal. Women refuse breast reconstruction because of their age, fear of another surgery, postoperative complications, recurrence of cancer, or financial reasons. Due to the low percentage of breast reconstruction procedures, there is a need to use external breast prostheses in patients after mastectomy (11–13).

In studies on the population of women after mastectomy, the most attention is paid to issues related to psychologic parameters, such as lack of acceptance of one's own body, reduced attractiveness, and sexuality (14–16). Some studies have also reported numerous functional disorders resulting mainly from an extensive wound, scarring, swelling, limited mobility in the joints of the shoulder girdle, and low muscle strength on the operated side (9, 13, 17).

Recently, post-mastectomy postural control disorders have become the subject of interest of many researchers (4, 9–11, 13, 18–27). The authors attempted to determine whether and how breast prosthesis affects body posture (28) and postural stability (11, 24). There is an agreement in both the existing literature and popular opinion that unilateral mastectomy results in postural control changes in women with breast cancer.

However, most of the abovementioned studies (4, 9–11, 13, 19, 25–27) assessed stability disorders in women after mastectomy by comparing them with their healthy counterparts. Considering the individual nature of regeneration of the body and primarily the course of compensation processes after treatment and unilateral breast amputation, it seems that the assessment of postural control disorders through inter-individual comparisons (women after mastectomy vs. healthy counterpart) may not be reliable.

Therefore, in this study, in addition to assessing the postural stability of women who underwent unilateral mastectomy, an attempt was made to evaluate the impact of external breast prosthesis (EBP) on postural stability by comparing the results of posturographic tests conducted on the same subject under two conditions—1) with EBP and 2) without EBP.

It has been hypothesized that postural stability disorders occur in women who undergo unilateral mastectomy and that the EBP plays a significant role in counteracting postural instability in this population. An additional aim of this study was to identify whether postural stability disorders in women who underwent unilateral mastectomy depended on the time since mastectomy was performed and time of using an EBP.

2 Materials and methods

A total of 52 women who underwent unilateral Modified Radical Mastectomy or Simple Mastectomy and who participated in the European Union program "You're worth it" were analyzed. The study was conducted in cooperation with the Gliwice Oncology Center and the Amazon Clubs of Zabrze and Gliwice. The program focused on providing comprehensive care and rehabilitation to women who had breast cancer living in the Silesian region. The average age of the participants was 61.8 ± 10.8 years (range: 38–84 years). The mean body weight of the patients was 78.4 kg, body length was 160.0 cm, and body mass index was 30.7. All participants had undergone combination therapy (unilateral Modified Radical Mastectomy or Simple Mastectomy, chemotherapy, and/or radiotherapy). In the study group, 27 women underwent left-sided mastectomy and 25 women underwent right-sided mastectomy. The mean time from surgery was 6.5 ± 7.6 years. All study participants wore an EBP, which was selected by a skilled person. All study participants used a EBP at least during the day. All study participants met the inclusion and exclusion criteria. The characteristics of the study group are presented in Table 1.

The inclusion criteria were as follows: 1) female sex, 2) unilateral Modified Radical Mastectomy or Simple Mastectomy, 3) use of EBP for at least 12 h during the day, and 4) provision of a written informed consent to participate in the study.

The exclusion criteria were as follows: 1) dizziness, 2) imbalance or use of medications affecting the body's balance, 3) nervous system diseases (Parkinson's disease, post-stroke condition, peripheral nerves paralysis), 4) system disorders and skeletal disorders (posture defects, foot deformities), 5) rheumatic diseases, 6) condition after injuries, 7) metastases to the skeletal system, and 8) mental disorders (depression, dementia).

Before initiating the study, each participant was informed of the purpose and assumptions of the research project and the individual elements of the study. Participants were also informed

TABLE 1 Characteristics of the participants.

Parameters	Mastectomy Group N=52		
	Mean ± SD	Median	Min–Max
Age (years)	61.8 ± 10.8	62.5	38 – 84
Height (cm)	160.0 ± 6.0	160.5	144 –173
Weight (kg)	78,4 ± 17.2	77.5	51–126
BMI (kg/m$^{2)}$	30.7 ± 6.6	29.4	17.4 – 47.3
Time since surgery (years)	6.5 ± 7.6	3.0	0.5 – 35.0

that participation in the study was completely voluntary and that it was possible to withdraw from the research project without providing any reason. The study was conducted after obtaining written consent from participants. The research design received a positive opinion from the Bioethical Committee of the Medical University of Silesia in Katowice (Resolution No. KNW/0022/KB1/61/18). The study was conducted in accordance with the Declaration of Helsinki.

The examination consisted of four interrelated parts—1) anthropometric measurements; 2) measurements of the circumference of the upper limbs; 3) evaluation of the weight-bearing distribution; and 4) posturographic testing (center of pressure [CoP] measurements).

2.1 Anthropometric measurements

The height and weight of the test person were measured using a scale with a height gauge. The length of the lower limbs was measured using a tensile-resistant sewing tape, measuring the distance between the greater trochanter of the femur and medial ankle separately for the right and left lower limbs.

2.2 Measurements of upper limb circumference

Upper limb circumferences were measured using a tensile-resistant sewing tape. Measurements were conducted at two levels in each participant. The first measurement (brachial circumference) was made approximately 10 **cm** above the lateral epicondyle of the humerus, while the second measurement (forearm circumference) was made approximately 10 **cm** below this epicondyle.

2.3 Measurements of weight-bearing distribution and posturographic tests (CoP measurements)

During posturographic examinations, the participants stood barefoot in a relaxed standing position, with the upper limbs along the body and head facing forward. The distance between the medial ankles was approximately 3 cm.

Posturographic examination was performed under twoconditions: 1) with the EBP and then 2) without the prosthesis. Successively, in both conditions following measurements were made:

1. Weight-bearing distribution between the A and NA sides of the body with the eyes open and then with eyes closed
2. Posturographic test with eyes open and then with eyes closed

A force plate PDM, ZEBRIS (Germany) with FootPrint software, was applied for posturographic examination. Each measurement was recorded three times (3 trial, each lasted for 30 seconds with 30 sec pauses between trials). The mean values from three trials were used for future analysis.

CoP shifts and surface area of the CoP were the basis for the following posturometric parameters:

1) Path length of the CoP (SPL)
2) Lateral sway path of the CoP—the length of the short axis of the ellipse – the width of the ellipse (WoE)
3) Anterior–posterior sway path of the CoP—the length of the long axis of the ellipse – the height of the ellipse (HoE)
4) Area of the ellipse containing 95% of the recorded points of the projection of the center of pressure into the ground (AoE)
5) Angle of the ellipse determined by CoP (aoE)

Flowchart describing the study flow is shown in Figure 1.

2.4 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 25. The data's statistical distribution was identified using the Shapiro–Wilk test, and descriptive statistics were calculated. The differences in the weight-bearing distribution, between the A and NA sides of the body, were expressed by the absolute values of the percent load of the A and NA sides and the

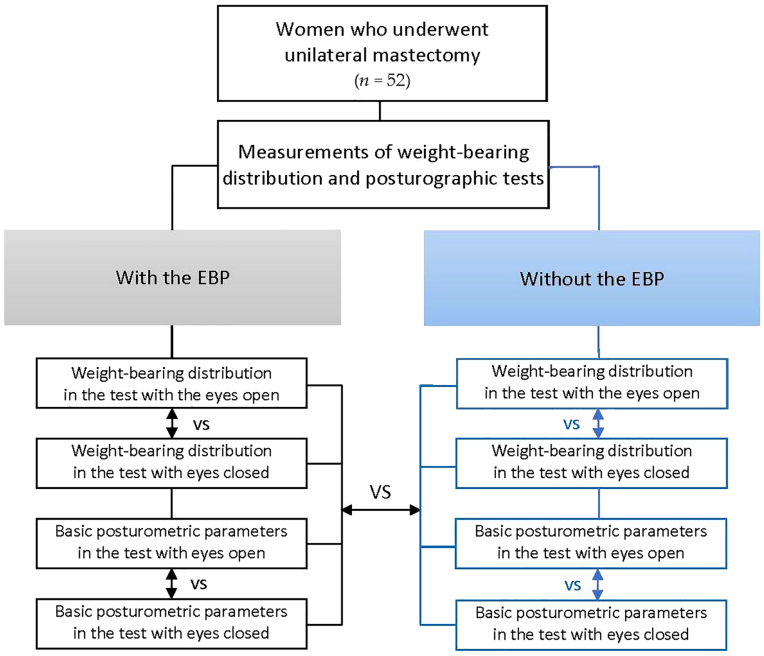


FIGURE 1
Flowchart describing the study flow. EBP, external breast prosthesis.

symmetry index (SI). SI was calculated using the following formula: $|X_{AM}-X_{NA}|/avg\ (X_{AM}, X_{NA}) \times 100\%$, where X_{AM} and X_{NA} are the values of a given parameter on both sides of the body – amputated (X_{AM}) and non-amputated (X_{NA}) (29). The differences in the parameters of the weight-bearing distribution between the A and NA sides of the body and posturometric parameters with and without the EBP, with the eyes open and closed, were analyzed using the Student's t-test or Wilcoxon's test. For the variables with a normal distribution, the Student's t-test was used for statistical calculations, while for variables with a distribution other than the normal, the Wilcoxon test was used for statistical calculations. The statistical significance level was considered at a P-value <0.05. The Mann–Whitney U test was used to identify differences in upper limb circumferences between the A and NA sides. Spearman's rank correlation test was used to calculate the correlation between the weight-bearing distribution and posturometric parameters and demographic data. Correlation coefficients were interpreted according to Altman's recommendations: Rs<0.2, weak; 0.21–0.4, low; 0.41–0.6, moderate; 0.61–0.8, high; and 0.81–1, very high.

3 Results

Measurement of the circumference of the upper limbs aimed to identify the presence of lymphedema in the upper limb on the amputated side. The comparison of the corresponding circumferences (brachial and forearm circumferences) between the A and NA limbs are summarized in Table 2.

The comparative analysis did not show any statistically significant differences in arm circumferences between the A and NA sides. However, significant differences were found in forearm circumferences. The mean difference in the forearm circumferences did not exceed 2 cm.

Based on the value of the SI, participants were divided into the following three subgroups:

A) SI ≥20—the percentage of load is greater on the amputated side
B) SI ≥-20—the percentage of load is greater on the non-amputated side
C) - 20< SI <20—the percentage of load is equal or close to equal on the A and NA sides

The analysis of the distribution of the participants in terms of the weight-bearing distribution between the A and NA sides showed that the dominant tendency was to load both sides of the body evenly. Regardless of the test conditions (with a prosthesis, without a prosthesis, with eyes open and eyes closed), in more than half of the participants, the weight-bearing distribution was equal or close to equal (less than 5% of the difference) between the A and NA sides (Table 3).

The results of the comparative analysis of the weight-bearing distribution between the A and NA sides of the body, expressed

TABLE 2 Differences in upper limb circumferences at the brachial level (circumference 1) and at the forearm level (circumference 2) between the A and NA sides.

Parameters	A			NA			p-value
	Mean ± SD	Median	Min–Max	Mean ± SD	Median	Min–Max	
circumference 1- brachial	23.42 ± 3.86	22	18 – 33	22.06 ± 3.64	22	17 – 29	0.156
circumference 2- forearm	30.44 ± 4.54	30	21 – 42	28.71 ± 3.64	28	21 – 39	**0.039**

p-value – statistical significance test value; statistically significant differences are printed in bold; A, amputated side; NA, non-amputated side.

as the percentage of load on the A and NA side, and the SI in both tested conditions, i.e., with and without the EBP, as well as with eyes open and closed, showed no statistically significant differences between the test results performed with and without an external prosthesis (Tables 4, 5).

No statistically significant differences have been observed between the results of posturographic tests conducted with and without an EBP during tests with eyes open and closed (Table 6). The only significant difference was in the angle of the ellipse defined by the projection of the center of gravity. In the test with EBP, the angle of the ellipse showed negative values, while, in the test without prosthesis, it showed positive values. Negative values of the ellipse angle indicate that the resultant of the gravity forces runs diagonally toward the left. Conversely, positive values of this angle indicate that the resultant of gravity forces runs diagonally toward the right (Table 6).

In comparing the posturometric parameters between the test conducted with eyes open and closed, in both conditions, i.e., with and without an EBP, statistically significant differences were noted in the path length of the CoP. The path of movement in the test conducted both with and without the EBP was statistically significantly longer in the test with eyes closed. Additionally, in the test conducted without the EBP, the HoE was significantly longer in the test with eyes closed. This indicates a significantly greater anterior–posterior CoP scavenging under conditions with visual inspection disabled (Table 7).

In analyzing the correlation between the weight-bearing distribution with demographic data (e.g., age of the participants, time since surgery, height, BMI, and body weight), no significant correlations were found. Exploration the relationship between posturometric parameters and demographic data revealed a several significant weak correlations. The following posturometric parameters: SPL, WoE, HoE and AoE were positively related to age of participants, which means, that the older participant, the worse the posturometric parameters (Table 8).

4 Discussion

This study aimed to assess the impact of EBP on postural stability of women who underwent unilateral mastectomy by comparing the results of the posturographic tests conducted on the same patient under two conditions, i.e., with and without EBP. Although the initial hypothesis that the EBP plays a significant role in maintaining a stable standing posture in women who underwent unilateral mastectomy seemed obvious and significant differences were expected, the study suggests the opposite. Not only did the conducted statistical analysis not confirm the differences in the weight-bearing distribution between the A and NA sides of the body in natural conditions, i.e., with EBP (as already presented above), there were also no significant differences between the tests conducted with and without the EBP. Moreover, in comparing the measurements of postural stability while maintaining a standing position with and without the EBP, no significant differences in the basic posturometric parameters were found, neither in the tests with open eyes nor in the tests with eyes closed.

Additionally, the differences in the arm circumferences on the A and NA sides did not confirm the occurrence of lymphedemas typical for the amputated limb after mastectomy. The hypothesis that the EBP provides better postural stability in women after unilateral mastectomy – in

TABLE 3 Characteristics of the symmetry index in individual trials.

Parameters	SI					
	eyes open			eyes closed		
	Mean ± SD	Median	Min–Max	Mean ± SD	Median	Min–Max
with EBP	-2.15 ± 17.33	-4	-48 – 48	-0.54 ± 16.84	2	-48 – 72
without EBP	0.38 ± 19.42	2	-56 – 56	1.54 ± 18.29	4	-56 – 48

EBP, external breast prosthesis; SI, symmetry index.

TABLE 4 Comparison of the weight-bearing distribution between the A and NA sides during the trial with and without the EBP while maintaining a free-standing position with the eyes open and closed (N = 52).

Parameters	eyes open		eyes closed		eyes open	eyes closed
	with EBP Mean ± SD	without EBP Mean ± SD	with EBP Mean ± SD	without EBP Mean ± SD	p-value	p-value
load on the A (%)	49.46 ± 4.33	50.10 ± 4.86	49.87 ± 4.21	50.38 ± 4.57	0.327*	0.346*
load on the NA (%)	50.54 ± 4.33	49.90 ± 4.86	50.13 ± 4.21	49.62 ± 4.57	0.438	0.346*
SI	13.08 ± 11.43	15.31 ± 11.71	11.62 ± 12.10	13.54 ± 12.25	0.143	0.397

*Student's t-test; p-value – statistical significance test value; EBP, external breast prosthesis; SI, symmetry index; A, amputated side; NA, non-amputated side.

the context of the obtained results – was not confirmed by the conducted studies.

The question whether EBP influences the postural stability of women who underwent mastectomy was not answered by the previous studies. Moreover, the literature on the subject indicates that the EBP does not play such a significant role as it could be assumed in the postural control of women who underwent unilateral mastectomy (11, 28, 30). Karczewska et al., based on studies assessing the influence of external prostheses on the dynamic balance of women who underwent unilateral mastectomy, found that the breast prosthesis did not affect the quality of equivalent reactions in the dynamic study (24). Similarly, in the extensive study conducted by Manikowska et al., who assessed the impact of external breast prostheses of three different weights on the measures of stability in women who underwent unilateral mastectomy, no statistically significant differences were found between posturographic tests conducted with different prostheses and without a breast prosthesis. It was interesting that the values of the weight-bearing distribution and posturometric parameters with the prosthesis, the mass of which was 50% of the amputated breast mass, were closest to the results obtained in the control group consisting of healthy counterparts (11). While examining the influence of EBP of different weights on the activity of the extensor muscles of the spine on the A and NA sides, Hojan et al. also found that the weight of the EBP did not affect the body posture of these women (28). In their subsequent studies, they presented scientific evidence that the weight of the EBP did not affect the biomechanics of the torso (30). Both the previous and presented results may indicate the activity of compensatory mechanisms activated as a result of the breast amputation procedure and organism's efforts to compensate for the postural symmetry disorder and its control.

Another goal of this study was to assess the dynamic postural stability of women who underwent unilateral mastectomy, a comparison of the stability measures in the posturographic test with eyes open and test excluding visual control in a standing position. Comparison of the postural stability measurements while maintaining the standing position with the EBP in place (i.e., under natural conditions) revealed significant differences between their values recorded during the test under visual control conditions (test with eyes open) and its switch-off conditions (test with eyes closed). The basic posturometric parameter, i.e., SPL, was almost 10% longer when the visual control was turned off than that with the eyes open. Therefore, the results of this study may indicate impairment of dynamic postural control, i.e., under conditions of disabled visual control in women who underwent unilateral mastectomy. Moreover, the abovementioned findings are confirmed by the results of previous scientific studies. Głowacka-Mrotek et al., in studies conducted on a group of patients who underwent unilateral mastectomy, comparing them to a group of healthy women, noted statistically significantly worse results in terms of stability measurements for both open and closed eyes in the group of women who underwent mastectomy. In the posturographic test with eyes open, this concerned the following parameters: maximum back deviation, maximum forward deviation, average Y deviation, average Y velocity, path length,

TABLE 5 Comparison of the weight-bearing distribution between the A and NA sides during the test with eyes open and closed while standing alone with and without the EBP (N = 52).

Parameters	with EBP		without EBP		with EBP	without EBP
	eyes open Mean ± SD	eyes closed Mean ± SD	eyes open Mean ± SD	eyes closed Mean ± SD	p-value	p-value
load on the A (%)	49.46 ± 4.33	49.87 ± 4.21	50.10 ± 4.86	50.38 ± 4.57	0.394	0.662*
load on the NA (%)	50.54 ± 4.33	50.13 ± 4.21	49.90 ± 4.86	49.62 ± 4.57	0.394	0.662*
SI	13.08 ± 11.43	11.62 ± 12.10	15.31 ± 11.71	13.54 ± 12.25	0.328	0.185

*Student's t-test; p-value, statistical significance test value; EBP, external breast prosthesis; SI, symmetry index; A, amputated side; NA, non-amputated side.

TABLE 6 Comparison of the results of posturographic tests conducted with and without EBP during independent standing in a free standing position with eyes open and closed (N = 52).

Parameters	eyes open		eyes closed		open eyes	eyes closed
	with EBP Mean ± SD	without EBP Mean ± SD	with EBP Mean ± SD	without EBP Mean ± SD	p-value	p-value
SPL (cm)	67.70 ± 21.95	63.70 ± 14.74	73.78 ± 22.75	70.05 ± 18.54	0.260	0.116
WoE (cm)	2.15 ± 1.27	2.23 ± 1.91	2.01 ± 1.08	2.15 ± 2.14	0.572	0.920
HoE (cm)	4.07 ± 1.64	3.70 ± 1.43	4.39 ± 1.88	4.66 ± 1.87	0.227	0.141
AoE (cm²)	7.50 ± 6.85	6.62 ± 6.26	7.46 ± 6.00	8.87 ± 12.14	0.355	0.584
aoE (°)	-4.05 ± 17.37	5.37 ± 18.45	-3.40 ± 16.41	-1.59 ± 10.30	**0.001***	0.756

*Student's t-test; p-value, statistical significance test value; statistically significant differences are printed in bold; EBP, external breast prosthesis; SPL, path length of the CoP; WoE, width of the ellipse; HoE, height of the ellipse; AoE, area of the ellipse; aoE, the angle of the ellipse.

and path surface area (13). However, in the sample with the exception of visual inspection, it concerned maximum backward deviation, maximum forward deviation, mean Y deviation, and path length. Moreover, these results confirm our observations regarding significantly higher HoE (which corresponds to the maximum backward and forward deviation range) recorded in the test without EBP with eyes closed. More recently, Mangone et al. compared the dynamic stability of women who underwent unilateral breast amputation to that of their healthy counterparts. They found that the stability measurements obtained from women who underwent mastectomy are worse both under visual control and after its switching off. They noted significantly worse results in both length of the ellipse and area of the ellipse plotted by the CoP in the test with eyes closed (10). In turn, Montezuma et al. recorded significantly higher maximum CoP velocity in both the tests with eyes open and eyes closed in comparative studies of women who underwent mastectomy and a control group of healthy women. Additionally, these results were significantly worse when the visual inspection was turned off in both groups (4). The abovementioned observations were also confirmed in the literature review on balance and gait studies in women who underwent mastectomy compared to their healthy counterparts (26). Despite the fact that this project did not involve comparative studies with healthy counterparts, the abovementioned previous studies lead to the conclusion that the posturometric parameters obtained in this study differ from the results of the population of healthy women presented in other studies and indicate the presence of symptoms of postural instability in the studied population. In both the previous studies and our study, the stability measurements were correlated with the age of the participants. Most posturometric parameters were worse in older participants. Deterioration of postural stabilization is an indispensable element of the aging process and a physiological phenomenon (31).

It can be assumed that the postural control systems in patients who underwent unilateral mastectomy rely largely on the visual feedback needed to maintain an upright body posture. Therefore, disabling visual control may expose the imperfections of postural control based on proprioceptive mechanisms in women after mastectomy. Some scientific reports that assessed the effect of treatment adjunct to mastectomy, such as neurotoxic chemotherapy, indicate that they may induce symptoms of peripheral neuropathy and lead to disturbances or even loss of proprioception (32–35).

While previous studies referred to the stability measurements obtained in tests with EBP (e.g., of different weights), often with results obtained from healthy counterparts

TABLE 7 Comparison of the results of the posturographic tests with eyes open and closed during self-standing in a free standing position during the tests with and without EBP (N = 52).

Parameters	with EBP		without EBP		with EBP	without EBP
	eyes open Mean ± SD	eyes closed Mean ± SD	eyes open Mean ± SD	eyes closed Mean ± SD	p-value	p-value
SPL (cm)	67.70 ± 21.95	73.78 ± 22.75	63.70 ± 14.74	70.05 ± 18.54	**0.001**	**0.000**
WoE (cm)	2.15 ± 1.27	2.01 ± 1.08	2.23 ± 1.91	2.15 ± 2.14	0.336	0.923
HoE (cm)	4.07 ± 1.64	4.39 ± 1.88	3.70 ± 1.43	4.66 ± 1.87	0.210	**0.004**
AoE (cm²)	7.50 ± 6.85	7.46 ± 6.00	6.62 ± 6.26	8.87 ± 12.14	0.707	0.059
aoE (°)	-4.05 ± 17.37	-3.40 ± 16.41	5.37 ± 18.45	-1.59 ± 10.30	0.728	0.078

p- value, statistical significance test value; statistically significant differences are printed in bold; EBP, external breast prosthesis; SPL, path length of the CoP; WoE, width of the ellipse; HoE, height of the ellipse; AoE, area of the ellipse; aoE, the angle of the ellipse.

TABLE 8 Correlation of posturometric parameters with the age of the participants.

Posturometric parameters	Age of the participants							
	with EBP				without EBP			
	eyes open		eyes closed		eyes open		eyes closed	
	r	p-value	r	p-value	r	p-value	r	p-value
SPL (cm)	**0.31**	**0.025**	**0.40**	**0.003**	0.26	0.066	0.31	0.028
WoE (cm)	**0.30**	**0.033**	0.20	0.148	0.27	0.052	0.19	0.180
HoE (cm)	**0.31**	**0.025**	0.25	0.075	0.18	0.207	0.12	0.406
AoE (cm^2)	**0.37**	**0.007**	**0.30**	**0.033**	**0.32**	**0.019**	0.20	0.162
aoE (°)	-0.01	0.931	-0.02	0.883	0.15	0.288	0.06	0.693

r, Spearman's rank correlation coefficient; p-value, value of the correlation significance test; statistically significant differences are printed in bold; EBP, external breast prosthesis; SPL, path length of the CoP; WoE, width of the ellipse; HoE, height of the ellipse; AoE, area of the ellipse; aoE, the angle of the ellipse.

(independent sample comparisons), which could raise doubts regarding homogeneity of the studied groups, our study compared dependent samples, i.e., stability measurements obtained in the tests with the target breast prosthesis with which the patient functions on a daily basis and also without it.

Additionally, the tests conducted with and without the target EBP were compared under two conditions—with and without visual inspection. To the best of our knowledge, these are the first studies using such a methodology.

The study results show that 1) postural stability disorders occur in women who underwent unilateral mastectomy after switching off visual control and 2) EBP does not have a significant influence on the symmetry of loading on the A and NA sides of the body and on the postural stability of women after unilateral mastectomy.

Based on the obtained results, the following conclusion can be drawn: the deficiencies of postural control in women after mastectomy indicate the need to include proprioceptive training as an element of rehabilitation of women after mastectomy.

5 Limitations

Our study has some limitations. Although in the case of the strictly defined purpose of the presented study, it did not matter that much, the study population varied considerably in terms of age, time since mastectomy, and adjuvant treatment methods. Therefore, in further stages of the study, the abovementioned limitations will be considered.

However, the most important limitation was the not fully recognized condition of lymphedema in the upper limb on the amputation side. It is necessary to consider the natural differences between the weight of the upper limbs, e.g., dominant and non-dominant, and this has not been considered in the presented studies. Therefore, in follow-up studies, a segmental analysis of body mass composition, especially the percentage of water in the upper limbs, should be included.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Bioethical Committee of the Medical University of Silesia in Katowice (Resolution No. KNW/0022/KB1/61/18). The patients/participants provided their written informed consent to participate in this study.

Author contributions

AK and MD-S: Conceptualization and writing – original draft. AK and MD-S: Investigation. AK and RŁ: Data curation. AS: Visualization. All authors contributed to the article and approved the submitted version.

Acknowledgments

We would like to thank all the women who took part in the study and the team of Medical Clinic "Sanus" in Zabrze.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Didkowska J, Wojciechowska U. Nowotwory piersi w polsce i europie — populacyjny punkt widzenia. *NOWOTWORY J Oncol* (2013) 63(2):111–8.

2. Głowacka-Mrotek I, Sowa M, Nowikiewicz T, Siedlecki Z, Hagner W, Zegarski W. Foot posture in female patients 5 years after breast-conserving surgery: a case-control study. *Breast Cancer* (2018) 25(3):325–33. doi: 10.1007/s12282-018-0835-y

3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* (2011) 61(2):69–90. doi: 10.3322/caac.20107

4. Montezuma T, de Oliveira Guirro EC, de Oliveira Lima Leite Vaz MM, Vernal S. Changes in postural control in mastectomized women. *J Cancer Ther* (2014) 5(6):493–9. doi: 10.4236/jct.2014.56056

5. Wojciechowska U, Didkowska J, Michałek I, Olasek P, Ciuba A. *Nowotwory złośliwe w polsce w 2018 roku*. Warszawa: Narodowy Instytut Onkologii im. Marii Skłodowskiej-Curie Państwowy Instytut Badawczy (2020) 7–8 p.

6. Smaga A, Mikułowska M, Komorowska A, Flakiewicz B, Gryglewicz J. *Rak piersi w polsce – leczenie to inwestycja*. Warszawa: Uczelnia Łazarskiego (2014) 14–21 p.

7. Senkus E, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rutgers E, et al. Primary breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* (2015) 26(5):v8–30. doi: 10.1093/annonc/mdv298

8. Moo TA, Sanford R, Dang C, Morrow M. Overview of breast cancer therapy. *PET Clin* (2018) 13(3):339–54. doi: 10.1016/j.cpet.2018.02.006

9. Ahn SY, Bok SK, Song Y, Lee HW, Jung JY, Kim JJ. Dynamic body posture after unilateral mastectomy: a pilot study. *Gland Surg* (2020) 9(5):1235–43. doi: 10.21037/gs-20-466

10. Mangone M, Bernetti A, Agostini F, Paoloni M, De Cicco FA, Capobianco SV, et al. Changes in spine alignment and postural balance after breast cancer surgery: A rehabilitative point of view. *Biores Open Access* (2019) 8(1):121–28. doi: 10.1089/biores.2018.0045

11. Manikowska F, Ozga-Majchrzak O, Hojan K. The weight of an external breast prosthesis as a factor for body balance in women who have undergone mastectomy. *Homo* (2019) 70(4):269–76. doi: 10.1127/homo/2019/1114

12. Metcalfe K, Gershman S, Ghadirian P, Lynch HT, Snyder C, Tung N, et al. Contralateral mastectomy and survival after breast cancer in carriers of BRCA1 and BRCA2 mutations: retrospective analysis. *BMJ* (2014) 348:g226. doi: 10.1136/bmj.g226

13. Głowacka-Mrotek I, Tarkowska M, Nowikiewicz T, Hagner-Derengowska M, Goch A. Assessment of postural balance in women treated for breast cancer. *Med (Kaunas)* (2020) 56(10):505. doi: 10.3390/medicina56100505

14. Archangelo SCV, Sabino Neto M, Veiga DF, Garcia EB, Ferreira LM. Sexuality, depression and body image after breast reconstruction. *Clinics (Sao Paulo)* (2019) 74:e883. doi: 10.6061/clinics/2019/e883

15. Lovelace DL, McDaniel LR, Golden D. Long-term effects of breast cancer surgery, treatment, and survivor care. *J Midwifery Womens Health* (2019) 64(6):713–24. doi: 10.1111/jmwh.13012

16. Neto MS, de Aguiar Menezes MV, Moreira JR, Garcia EB, Abla LE, Ferreira LM. Sexuality after breast reconstruction post mastectomy. *Aesthetic Plast Surg* (2013) 37(3):643–7. doi: 10.1007/s00266-013-0082-8

17. Boquiren VM, Hack TF, Thomas RL, Towers A, Kwan WB, Tilley A, et al. A longitudinal analysis of chronic arm morbidity following breast cancer surgery. *Breast Cancer Res Treat* (2016) 157(3):413–25. doi: 10.1007/s10549-016-3834-8

18. Serel S, Tuzlalı ZY, Akkaya Z, Uzun Ç, Kaya B, Bayar S. Physical effects of unilateral mastectomy on spine deformity. *Clin Breast Cancer* (2017) 17(1):29–33. doi: 10.1016/j.clbc.2016.10.004

19. Rostkowska E, Bak M, Samborski W. Body posture in women after mastectomy and its changes as a result of rehabilitation. *Adv Med Sci* (2006) 51:287–97.

20. Barbosa Jde A, Amorim MH, Zandonade E, Delaprane ML. Avaliação da postura corporal em mulheres com câncer de mama [Evalution of body posture in women with breast cancer]. *Rev Bras Ginecol Obstet* (2013) 35(5):215–20. doi: 10.1590/s0100-72032013000500005

21. Głowacka I, Nowikiewicz T, Siedlecki Z, Hagner W, Nowacka K, Zegarski W. The assessment of the magnitude of frontal plane postural changes in breast cancer patients after breast-conserving therapy or mastectomy - follow-up results 1 year after the surgical procedure. *Pathol Oncol Res* (2016) 22(1):203–8. doi: 10.1007/s12253-015-9995-7

22. Atanes Mendes Peres AC, Dias de Oliveira Latorre MD, Yugo Maesaka J, Filassi JR, Chada Baracat E, Alves Gonçalves Ferreira E. Body posture after mastectomy: Comparison between immediate breast reconstruction versus mastectomy alone. *Physiother Res Int* (2017) 22(1):1–9. doi: 10.1002/pri.1642

23. Kabala M, Wilczynski J. Evaluation of equivalent reactions in women after mastectomy using biodex balance system. *J Education Health Sport* (2017) 7(12):565–78. doi: 10.5281/zenodo.1133958

24. Karczewska E, Szlachta P, Chamera S, Latosiewicz R. Evaluation of the dynamic balance in women after unilateral mastectomy and external breast prosthesis – a pilot study. *Curr Problems Biomech* (2017) 13:29–36.

25. Rachwał M, Drzał-Grabiec J, Walicka-Cupryś K, Truszczyńska A. Control of static balance among women after mastectomy. vision impact on the quality of the equilibrium response. *Adv Rehabil* (2013) 27(3):13–20. doi: 10.2478/rehab-2014-0016

26. Hsieh KL, Wood TA, An R, Trinh L, Sosnoff JJ. Gait and balance impairments in breast cancer survivors: A systematic review and meta-analysis of observational studies. *Arch Rehabil Res Clin Transl* (2019) 1(1-2):100001. doi: 10.1016/j.arrct.2018.12.001

27. Malicka I, Barczyk K, Hanuszkiewicz J, Skolimowska B, Woźniewski M. Body posture of women after breast cancer treatment. *Ortop Traumatol Rehabil* (2010) 12(4):353–61.

28. Hojan K, Manikowska F, Chen BP, Lin CC. The influence of an external breast prosthesis on the posture of women after mastectomy. *J Back Musculoskelet Rehabil* (2016) 29(2):337–42. doi: 10.3233/BMR-150648

29. Zifchock RA, Davis I, Higginson J, Royer T. The symmetry angle: a novel, robust method of quantifying asymmetry. *Gait Posture* (2008) 27(4):622–7. doi: 10.1016/j.gaitpost.2007.08.006

30. Hojan K, Manikowska F. Can the weight of an external breast prosthesis influence trunk biomechanics during functional movement in postmastectomy women? *BioMed Res Int* (2017) 2017:9867694. doi: 10.1155/2017/9867694

31. Viswanathan A, Sudarsky L. Balance and gait problems in the elderly. *Handb Clin Neurol* (2012) 103:623–34. doi: 10.1016/B978-0-444-51892-7.00045-0

32. Hile ES, Fitzgerald GK, Studenski SA. Persistent mobility disability after neurotoxic chemotherapy. *Phys Ther* (2010) 90(11):1649–57. doi: 10.2522/ptj.20090405

33. Kneis S, Wehrle A, Freyler K, Lehmann K, Rudolphi B, Hildenbrand B, et al. Balance impairments and neuromuscular changes in breast cancer patients with chemotherapy-induced peripheral neuropathy. *Clin Neurophysiol* (2016) 127(2):1481–90. doi: 10.1016/j.clinph.2015.07.022

34. Monfort SM, Pan X, Patrick R, Singaravelu J, Loprinzi CL, Lustberg MB, et al. Natural history of postural instability in breast cancer patients treated with taxane-based chemotherapy: A pilot study. *Gait Posture* (2016) 48:237–42. doi: 10.1016/j.gaitpost.2016.06.011

35. Wampler MA, Topp KS, Miaskowski C, Byl NN, Rugo HS, Hamel K. Quantitative and clinical description of postural instability in women with breast cancer treated with taxane chemotherapy. *Arch Phys Med Rehabil* (2007) 88(8):1002–8. doi: 10.1016/j.apmr.2007.05.007

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 05 September 2022
DOI 10.3389/fonc.2022.886831

OPEN ACCESS

EDITED BY
Mangesh A. Thorat,
Guy's and St Thomas' NHS Foundation Trust,
United Kingdom

REVIEWED BY
Robert Wesolowski,
The Ohio State University,
United States
Francesco Schettini,
Institut de Recerca Biomèdica August Pi i Sunyer (IDIBAPS), Spain

*CORRESPONDENCE
Katya Galactionova
e.galactionova@unibas.ch

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 01 March 2022
ACCEPTED 08 August 2022
PUBLISHED 05 September 2022

CITATION
Galactionova K, Loibl S, Salari P, Marmé F, Martin M, Untch M, Bonnefoi HR, Kim S-B, Bear HD, McCarthy N, Gelmon KA, García-Sáenz JA, Kelly CM, Reimer T, Toi M, Rugo HS, Gnant M, Makris A, Burchardi N and Schwenkglenks M (2022) Cost-effectiveness of palbociclib in early breast cancer patients with a high risk of relapse: Results from the PENELOPE-B trial. *Front. Oncol.* 12:886831. doi: 10.3389/fonc.2022.886831

Cost-effectiveness of palbociclib in early breast cancer patients with a high risk of relapse: Results from the PENELOPE-B trial

Katya Galactionova[1]*, Sibylle Loibl[2], Paola Salari[1], Frederik Marmé[3], Miguel Martin[4,5], Michael Untch[6], Hervé R. Bonnefoi[7], Sung-Bae Kim[8], Harry D. Bear[9], Nicole McCarthy[10,11], Karen A. Gelmon[12], José A. García-Sáenz[13,14], Catherine M. Kelly[15], Toralf Reimer[16], Masakazu Toi[17], Hope S. Rugo[18], Michael Gnant[19], Andreas Makris[20], Nicole Burchardi[2] and Matthias Schwenkglenks[1]

[1] Institute of Pharmaceutical Medicine (ECPM), University of Basel, Basel, Switzerland, [2]German Breast Group, Neu-Isenburg, Germany, [3]Medical Faculty Mannheim, Heidelberg University, University Hospital Mannheim, Mannheim, Germany, [4]Instituto de Investigacion Sanitaria Gregorio Marañon, Centro de Investigación Biomédica en Red Cáncer (CIBERONC), Universidad Complutense, Madrid, Spain, [5]Spanish Breast Cancer Group, Grupo Español de Investigación en Cáncer de Mama (GEICAM), Madrid, Germany, [6]Department of Obstetrics and Gynaecology, Helios Kliniken Berlin-Buch, Berlin, Germany, [7]Department of Medical Oncology, Institut Bergonié and Université de Bordeaux Institut National de la Santé et de la Recherche Médicale (INSERM) U916, Bordeaux, France, [8]Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea, [9]Division of Surgical Oncology, Massey Cancer Center, Virginia Commonwealth University, Virginia Commonwealth University (VCU) Health, Richmond, VA, United States, [10]Australia and New Zealand Breast Cancer Trials Group, Newcastle, NSW, Australia, [11]Department of Medical Oncology, University of Queensland, Brisbane, QLD, Australia, [12]BC Cancer, Vancouver, BC, Canada, [13]Instituto de Investigación Sanitaria del Hospital Clinico San Carlos (IdISSC), Madrid, Spain, [14]Grupo Español de Investigación en Cáncer de Mama (GEICAM), Madrid, Spain, [15]Mater Private Hospital, Cancer Trials Ireland, Dublin, Ireland, [16]Department of Obstetrics and Gynecology, University of Rostock, Rostock, Germany, [17]Breast Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan, [18]University of California San Francisco Comprehensive Cancer Center, San Francisco, CA, United States, [19]Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria, [20]Institute of Cancer Research, London, United Kingdom

Background: Patients with hormone receptor-positive, HER2-negative breast cancer who have residual invasive disease after neoadjuvant chemotherapy (NACT) are at a high risk of relapse. PENELOPE-B was a double-blind, placebo-controlled, phase III trial that investigated adding palbociclib (PAL) for thirteen 28-day cycles to adjuvant endocrine therapy (ET) in these patients. Clinical results showed no significant improvement in invasive disease-free survival with PAL.

Methods: We performed a pre-planned cost-effectiveness analysis of PAL within PENELOPE-B from the perspective of the German statutory health insurance. Health-related quality of life scores, collected in the trial using the EQ-5D-3L

instrument, were converted to utilities based on the German valuation algorithm. Resource use was valued using German price weights. Outcomes were discounted at 3% and modeled with mixed-level linear models to adjust for attrition, repeated measurements, and residual baseline imbalances. Subgroup analyses were performed for key prognostic risk factors. Scenario analyses addressed data limitations and evaluated the robustness of the estimated cost-effectiveness of PAL to methodological choices.

Results: The effects of PAL on quality-adjusted life years (QALYs) were marginal during the active treatment phase, increasing thereafter to 0.088 (95% confidence interval: −0.001; 0.177) QALYs gained over the 4 years of follow-up. The incremental costs were dominated by PAL averaging EUR 33,000 per patient; costs were higher in the PAL arm but not significantly different after the second year. At an incremental cost-effectiveness ratio of EUR 380,000 per QALY gained, PAL was not cost-effective compared to the standard-of-care ET. Analyses restricted to Germany and other subgroups were consistent with the main results. Findings were robust in the scenarios evaluated.

Conclusions: One year of PAL added to ET is not cost-effective in women with residual invasive disease after NACT in Germany.

KEYWORDS

Penelope-B, Palbociclib, CDKi, (postneo)adjuvant, early breast cancer, cost-effectiveness, Germany

Introduction

Breast cancer is the most frequent cancer among women in Europe. In 2020, about 355,000 new cases were diagnosed, with nearly 95,000 women dying of breast cancer in EU-27 that year (1). About 90% of new breast cancer patients in EU-27 countries are diagnosed at an early stage (2), of which, approximately a third will develop advanced or metastatic disease later in life (3). Prognosis depends on the number of positive axillary nodes, tumor size, tumor grade, lymphatic and vascular invasion, expression of estrogen (ER+) and progesterone receptors, and human epidermal growth factor 2 (HER2) status (4, 5).

In recent years, novel cancer treatments led to patient-relevant improvements in treatment outcomes. In particular, cyclin-dependent kinase (CDK) 4/6 inhibitors, including palbociclib (PAL), combined with endocrine therapy (ET) showed impressive efficacy in ER+ advanced breast cancer (6–8). In hormone receptor-positive breast cancer patients, CDKs modulate cell cycle entry and progression in response to growth signals (9, 10). Inhibition of these kinases with PAL could enhance the activity of other anticancer drugs.

PENELOPE-B follows a series of studies that established the efficacy of PAL in metastatic breast cancer (11). In 2015, the US FDA and, later, also the EMA approved PAL for use in combination with ET for first-line and, subsequently, second-line treatment of postmenopausal women with locally advanced or metastatic disease (12, 13). Since then, several recent and ongoing trials, including PENELOPE-B, have sought to demonstrate its efficacy also in high-risk ER+, HER2-, early breast cancer patients with residual disease after neoadjuvant chemotherapy (NACT) (14–17).

In PENELOPE-B, PAL added to standard adjuvant ET did not statistically improve invasive disease-free survival (iDFS) or overall survival (OS) compared to placebo (16). Findings on these patient-level outcomes expanded the clinical evidence base but were not sufficient to conclude on the value of PAL against competing claims for healthcare resources (18). These value judgments, addressed within the cost-effectiveness framework, integrate societal health state values through the use of preference-based health-related quality of life (HRQoL) measures and costs, thus reflecting efficiency and equity (18, 19). An intervention that does not lead to a meaningful benefit in terms of survival may nonetheless be a good value for money if it leads to a better HRQoL or changes in care-seeking that reduce overall spending in the patient group targeted. Toward this end, we present further, pre-planned analyses of the trial data on the effects of PAL on HRQoL, medical resource use, and cost of care, and address its cost-effectiveness compared to ET alone.

Materials and methods

We performed a within-trial cost-effectiveness analysis of PAL+ET in PENELOPE-B from the perspective of the German statutory health insurance. Information on survival, disease progression, medical resource use, and HRQoL based on the European Quality of Life-5 Dimensions-3 Level (EQ-5D-3L) instrument was collected within the trial. Price weights were obtained from published national databases and the literature (20–25). A validated German valuation algorithm for ED-5D-3L was used to derive quality-adjusted life years (QALYs) (26). Mixed-level models (27) were used to adjust for missing values, stratification, and potential residual imbalances between the study arms at baseline. The evaluation was restricted to a within-trial horizon with a maximum follow-up (FU) of up to 6 years. Incremental costs and effects, discounted at 3%, were compared in each year and cumulatively over the duration of FU. Scenario analyses addressed data limitations and evaluated the robustness of the estimated cost-effectiveness of PAL to methodological choices. The main analysis was conducted on the intent-to-treat (ITT) population; subgroup analyses including by risk strata and country were also performed.

Trial

PENELOPE-B (NCT01864746) was a randomized, double-blind, placebo-controlled, phase III trial that investigated the effects of PAL in early HR+ and HER2- breast cancer patients aged 18 and above (28). Women were eligible if they had residual disease after at least 16 weeks of NACT, were at a high risk of relapse [clinical pathological staging-estrogen receptor grading (CPS-EG) score ≥ 3 or 2 and ypN+ (29)], and subsequently underwent a definitive surgery and/or radiation.

Patients were recruited between February 2014 and December 2017 from 221 centers in Germany, Spain, USA, France, Australia, South Korea, Ireland, Japan, Austria, and UK. Randomization was in 1:1 permuted blocks of alternating size stratified by risk, nodal involvement after surgery, Ki-67 status, age, and region to receive either PAL (125 mg, orally, once daily for 21 days, followed by 1 week off treatment for a total duration of thirteen 4-week cycles) or placebo in addition to adjuvant ET and other standard-of-care treatment according to local guidelines (28). Patients were followed up for a maximum of 6 years. The primary clinical end point of the trial was iDFS.

The trial was approved by the health authorities and ethics committees and conformed to ICH-GCP guidelines and the Declaration of Helsinki. Further details on the trial are available from Loibl et al. (16).

End point

The primary end point for the health-economic sub-study was the incremental cost-effectiveness of PAL+ET expressed as a cost per QALY gained compared to ET (implies placebo+ET here and throughout). The secondary objective was to compare between the arms HRQoL, accrued QALYs, medical resource use, and direct medical costs. The outcomes were assessed yearly and cumulatively within the trial FU. No extrapolation was done due to the lack of clinical differences between the trial arms at the end of FU.

QALYs

The EQ-5D-3L (30, 31) was used to score HRQoL. The questionnaire, asking patients to rank their mobility, self-care, usual activities, pain/discomfort, and anxiety/depression, was completed at baseline (30 days prior to randomization), and during FU visits: bi-monthly during PAL treatment, at end of treatment (EOT), every 6 months in years 2–4, and every 12 months thereafter. The EQ-5D-3L scores were converted to utilities using the German valuation algorithm (26). QALYs were estimated by combining the estimated utilities with time using the "area under the curve" approach (32). For patients who died in the trial, QALYs were set to 0 from the date of death until the end of planned FU.

Medical resource use, price weights, and costs

Medical resource use recorded in the trial covered all care episodes including those related to conditions other than breast cancer. Care episodes occurring at the enrolling and treating medical centers were transferred from the patients' medical records. Patient diaries were used as the basis for recording the intake of the study drug and care episodes (outpatient physician visits and hospitalizations) occurring outside of the enrolling and treating centers. Information on the medical resource use generally allowed characterization of care episodes with respect to the type of care received, the number of events since last FU, and their duration facilitating costing. Where information was recorded as free text (diagnostic screenings, physician visits, and hospitalizations), coding routines were developed to map these entries into line items that could be consistently costed. See Appendix A1 for further details on recording of resource use in the trial and adjustments for costing.

2020 German price weights (i.e., unit costs) were used to value resources. Drug prices were based on the median listed retail price per tablet (20). Costs of radiotherapy per session were obtained from the literature (24). Physician visits were costed by specialty based on the average fee reported by the National Association of Statutory Health Insurance Physicians [Kassenärztliche Bundesvereinigung (KBV)] (21); 2018 unit costs (the most recent available at the time of analysis) were inflated to 2020 prices using the German gross domestic product deflator (33). Screenings and other diagnostic examinations (i.e., CT, MRI, mammogram), minor surgeries (i.e., biopsy), and lymph drainage massage were also costed from KBV data (22). Physiotherapy costs were based on costs of inpatient hospital rehabilitation from the German Pension Insurance (Deutsche Rentenversicherung) (23). Inpatient hospital stays were costed based on an average cost per day for different types of hospitalization derived by dividing the average cost per stay by the average length of stay from appropriate diagnosis-related groups (DRGs) in the German DRG system (25). See Appendix A1 for unit costs and further details on derivation.

Only resources used after randomization (including postoperative treatments, care related to comorbidities, adverse events, and treatments for recurrent or secondary malignancies) were considered. Costs were calculated per care episode by multiplying the quantity of the resource line item with the respective price weight and then summed. For patients who died during FU, costs were set to 0 from the date of death until the end of planned FU.

Missing values

Missing values were encountered due to partial response (item-level missingness), attrition (loss to FU or withdrawal from the study), and, to the extent that we produced estimates for a given length of FU (as opposed to average FU), missingness due to administrative censoring (i.e., patients followed up for less than 72 months given their date of enrolment). Each of these sources of missing information required its own strategy to address.

Instances of item-level missingness were relatively few, resulting in <1% of missing values in utility and resource use data. These were resolved with information borrowed from the available data or filled with assumptions informed by clinical experts (see Table A4 in Appendix A1 for details). Care episodes that took place in the periods of missed FU were deemed not to pose a significant problem for costs since these were covered during the next FU visit at which the patient was present (i.e., medical records reviewed and relevant information updated since the last visit). Missing utility values were linearly interpolated from the periods just before the missing value and just after. Missing baseline utility values were imputed from the FU visit at the start of the first treatment cycle since the two were on average <30 days apart (patients have only received the first dose of study drug in-between).

Patients lost to FU for multiple consecutive periods were censored at the date of the last FU present; data in subsequent periods in which FU was resumed were not used (<2% of patient-FU records). For patients who died, costs were censored at the time of the previous FU when the patient was alive to reflect missing information on expenditures prior to death. For consistency, data used in regression analyses were further censored to exclude partial year entries, i.e., censoring at the last complete yearly interval.

In total, attrition resulted in 20% of QALYs and 11% of costs missing (see Table A6 in Appendix A1). Attrition was balanced between the study arms and increased from about 13% for QALYs and 7% for costs in the first year to as much as 23% and 38%, respectively, in year 3 before dropping again in the later years. Administrative censoring accounted for another 23% of missing values. Missingness varied by country and was strongly and positively associated with the time of enrollment and CPS-EG score ≥3 (Tables A7–A9 in Appendix A1).

Between-country heterogeneity

We tested and found no evidence of heterogeneity in outcomes among countries (see Table A10 in Appendix A1) (34–36). Thus, the pooled result applies to all countries that participated in the trial, including Germany.

Cost-effectiveness analysis

The main analysis was conducted on the ITT population at 4 years after randomization; results for years 5 and 6 could not be reliably estimated due to high administrative censoring.

As the starting point for the main analysis, mean differences in outcomes were calculated by arm and year of FU. These descriptive results were then compared to regression-adjusted mean differences estimated with mixed models for repeated measures (MMRM) (27). The models adjusted for stratification (37) and addressed missingness under the missing at random assumption (MAR) (27, 38) and potential residual imbalances at baseline (32). The effect of PAL was captured with an interaction between the arm assignment and the year of FU. We modeled residuals using an unstructured covariance matrix that implies independence between patients. All models controlled for risk stratification factors, baseline health utility, and country; cost models additionally controlled for breast cancer treatments received before randomization (first ET with tamoxifen, ovarian ablation with goserelin injections, mastectomy, and reconstruction surgery) and the number of health conditions with ongoing treatment (0, 1, 2, 3, and more). Average marginal effects by year were summed to produce cumulative incremental

outcomes for different lengths of FU. Model specification tests are reported in Tables A11 and A12 in Appendix A1. Alternative specifications, allowing for correlation between outcomes using seemingly unrelated regressions (SUR) and adjusting for skewness using generalized linear models (GLMs), were tested in scenario analyses.

The incremental cost-effectiveness ratio (ICER) was calculated as the ratio of cumulative incremental costs to QALYs.

Uncertainty and scenario analyses

The 95% confidence intervals for the incremental QALYs and costs were estimated with nonparametric bootstrap stratified by arm with 5,000 replications. Confidence intervals and *p*-values were calculated by pooling bootstrapped standard errors over the respective yearly intervals. Regression-adjusted bootstrapped incremental outcomes were plotted on the cost-effectiveness plane.

Scenario analyses addressed data limitations and evaluated the robustness of the estimated cost-effectiveness of PAL to methodological choices and in different populations of interest. We tested our strategy for dealing with missing values by relaxing some of the censoring rules and by using multiple imputation by chained equations (MICE) to impute missing values for each FU year (see Appendix A2 for details on the implementation of MICE) (39, 40). Analyses using imputed data allowed us to further explore between- and within-patient correlations (38).

Technical implementation

All analyses were implemented in Stata/SE version16.1 (41).

Results

Patient characteristics

The PENELOPE-B study population was previously described in Loibl et al. (16). For context, patient characteristics are reported in Table A13 in Appendix A3. We briefly note that Germany recruited over a third of all patients. These differed somewhat from the full study population in the distribution of risk (relatively higher share with CPS-EG score ≥3 in PAL+ET arm, lower with Ki-67 ≤15%), breast cancer treatments at baseline (fewer mastectomies, fewer started ET before PAL or ET with tamoxifen, fewer on goserelin, and fewer hysterectomies), and other illnesses (one illness less chronic or ongoing).

Descriptive results

Clinical results have been previously reported in Loibl et al. (16). To facilitate the interpretation of incremental effects of PAL on HRQoL and costs, we present unadjusted clinical events along with healthcare utilization summaries (Table 1). On average, about 23% of patients relapsed, less than 2% developed a secondary malignancy, and about 10% died during the FU period. These fractions were relatively higher in the German subpopulation, reflecting longer FU and differences in baseline characteristics. In both samples, the fraction reporting an event was higher in the ET arm compared to PAL+ET, although this difference was not statistically significant.

Most patients had at least one screening, one visit with a physician, and had taken at least one hormone therapy pill. About half had a hospitalization, and about a quarter received additional ovarian suppression injections. Other types of care were less common. Consistent with differences in clinical events, a higher fraction of patients in the German subpopulation were hospitalized, received targeted therapy, mental health services, and physiotherapy compared to the full study population. With the exception of PAL, there were no statistical differences between the arms in healthcare utilization. Furthermore, mean quantities by type of care were balanced except for PAL and physician visits (about five more in PAL+ET, incurred in the first year; see Table A14 and Figures A7–A12 in Appendix A3 for additional tabulations).

In the full study population, over the average FU of about 2.8 years, patients in the PAL+ET arm gained an additional 0.07 discounted QALYs compared to the ET arm, and this difference was not significant (Table 2). In the German subpopulation, the relative gains were larger (0.23 discounted QALYs) and marginally significant. Differences in total costs were statistically significant and roughly equal to the average cost of 13 months of PAL (EUR 35,000). The second-largest contributor to total costs was hospitalizations, averaging about EUR 2,000 per patient (note that only about 50% had any hospitalizations and about 40% were followed up for more than 4 years), followed by physician visits, and injections for ovarian suppression. In the ET arm, the cost distributions were fairly similar with the exception of PAL and physician visits—patients in PAL+ET arm spent, on average, EUR 500 more on physician visits over the FU period

Regression-adjusted results

The mean yearly differences in outcomes between study arms were cumulated in Table 3 to show the total health-economic effects of PAL throughout the trial FU. Unadjusted effects of PAL on QALYs were marginal during the active

TABLE 1 Clinical events and utilization.

Population	All countries			Germany		
Arm	**PAL + ET (*N* = 631)**	**ET (*N* = 619)**	***p*-value**	**PAL + ET (*N* = 218)**	**ET (*N* = 214)**	***p*-value**
			Clinical events			
FU, years[a]	4.13 ± 0.84	4.13 ± 0.84	0.958	4.35 ± 0.98	4.38 ± 0.95	0.802
Relapsed, %	22.7	23.3	0.801	29.2	31.6	0.586
Number of relapses, *n*	1.63 ± 1.06	1.68 ± 1.22	0.971	1.54 ± 1.06	1.73 ± 1.06	0.148
Developed a secondary malignancy, %	1.6	1.8	0.791	1.8	1.9	0.979
Died, %	9.8	11.1	0.446	16.4	17.2	0.830
			Any service use by type, %			
PAL	99.7	0.8	<0.001	99.5	0.5	<0.001
Hospitalization	45.4	44.7	0.818	51.4	50.7	0.888
Screening	98.9	99.2	0.583	99.5	98.6	0.308
Physical examinations and specialist visits[b]	99.8	99.8	0.990	100.0	99.5	0.313
Targeted therapy	8.6	11.0	0.151	10.1	14.4	0.170
Hormone therapy	100.0	99.8	0.313	100.0	100.0	<0.001
Ovarian suppression[c]	23.3	25.5	0.367	15.1	20.0	0.184
Radiation therapy	5.7	5.8	0.939	7.3	8.4	0.690
Chemotherapy	13.2	10.7	0.171	15.1	14.9	0.941
Mental health or physiotherapy	4.0	4.2	0.836	9.2	7.9	0.637
			Of those with any service use number of visits/days of therapy/number of pills, *n*			
PAL	324 ± 95	336 ± 63	0.947	325 ± 93	364 ± .[d]	0.604
Hospitalizations	7 ± 13	7 ± 11	0.828	9 ± 10	11 ± 12	0.135
Screening	7 ± 5	7 ± 4	0.164	8 ± 5	7 ± 5	0.104
Physical examinations and specialist visits[b]	33 ± 12	29 ± 11	<0.001	34 ± 13	29 ± 13	<0.001
Targeted therapy	172 ± 133	196 ± 154	0.369	186 ± 138	210 ± 183	0.655
Hormone therapy	766 ± 397	769 ± 421	0.821	747 ± 398	794 ± 463	0.143
Ovarian suppression[c]	19 ± 17	18 ± 17	0.641	13 ± 11	11 ± 12	0.281
Radiation therapy	17 ± 12	19 ± 13	0.531	18 ± 10	22 ± 15	0.557
Chemotherapy	228 ± 196	280 ± 212	0.054	201 ± 147	309 ± 222	0.046
Mental health or physiotherapy	32 ± 41	17 ± 13	0.349	37 ± 44	23 ± 10	0.924

Continuous variables are summarized, with a mean ± SD. Significance of differences in the number of clinical events and care episodes between the arms was assessed with Fisher's exact test for binary, continuity-corrected chi-square test for categorical, and Wilcoxon test for continuous parameters. [a]FU refers to the number of years between patient entry date and study end date irrespective of event; [b]Excluding visits related to administration of ovarian suppression, including examinations by physicians, referral, and follow-up visits related to screenings and hospitalizations; [c]Included goserelin or other luteinizing hormone-releasing hormone (LHRH) injections but not surgery or radiotherapy that were covered under the respective event types; [d]SD missing since only one patient received PAL in ET arm in Germany.
ET, endocrine therapy; FU, follow-up; PAL, palbociclib.

treatment phase and increased, favoring PAL, in later years, leading to a sizable and statistically significant effect over years 1–4 (see also Tables A15 and A16 in Appendix A3 for yearly incremental differences). Regression-adjusted estimates of PAL impacts on QALYs were not significant and less favorable than the descriptive result, while the upward time trend was numerically maintained. The cumulative effect over FU years 1–4 added up to 0.09 QALYs gained and was marginally significant. Impacts on costs aligned with the descriptive result, with the bulk of incremental costs accrued in the first year; these increased only marginally throughout FU. The estimated ICER was about EUR 380,000 per QALY gained, which is nearly double the unadjusted ratio, consistent with differences in effectiveness.

Uncertainty and scenario analysis

Figure 1 presents the probabilistic distribution of regression-adjusted incremental outcomes cumulated over years 1–4 from a bootstrap resampling with 5,000 replications. The plot highlights the great extent to which the uncertainty in the cost-effectiveness of PAL was driven by uncertainties on its effects on QALYs.

Key scenarios, testing the robustness of the main result, are presented in Table 4 (see Appendix A4 for the full set). Analyses restricted to patients recruited in Germany (scenario 1) yielded a lower point estimate on QALYs with no statistical significance and an estimated ICER exceeding EUR 1,600,000. Otherwise, effects on QALYs were marginally significant across scenarios evaluated [positive in all but the complete case analysis (scenario

TABLE 2 Quality-adjusted life years and costs.

Population	All countries			Germany		
Arm	**PAL + ET (*N* = 631)**	**ET (*N* = 619)**	***p*-value**	**PAL + ET (*N* = 218)**	**ET (*N* = 214)**	***p*-value**
		Quality of life, n				
FU, years[a]	2.82 ± 1.39	2.74 ± 1.3	0.152	3.03 ± 1.48	2.69 ± 1.44	0.012
Missing[b] QALYs, %	42.1	42.9	0.042	37.3	42.1	0.192
Baseline utility	0.90 ± 0.13	0.89 ± 0.14	0.205	0.91 ± 0.12	0.90 ± 0.12	0.464
Total QALYs	2.50 ± 1.31	2.42 ± 1.22	0.188	2.65 ± 1.40	2.39 ± 1.33	0.054
Total discounted QALYs	2.34 ± 1.20	2.27 ± 1.12	0.188	2.47 ± 1.28	2.24 ± 1.22	0.055
		Costs, EUR				
FU, years[a]	3.38 ± 1.16	3.28 ± 1.00	0.060	3.46 ± 1.30	3.29 ± 1.00	0.111
Missing[b] costs, %	33.6	34.8	0.353	30.7	32.7	0.617
PAL	33,193 ± 9,921	279 ± 3,138	<0.001	33,233 ± 9,812	175 ± 2,557	<0.001
Hospitalization	2,272 ± 7,450	2,302 ± 6,605	0.706	3,044 ± 5,198	4,048 ± 7,508	0.661
Screening	374 ± 437	338 ± 352	0.474	334 ± 315	323 ± 320	0.386
Physical examinations and specialist visits[c]	1,912 ± 805	1,457 ± 574	<0.001	1,947 ± 885	1,454 ± 674	<0.001
Targeted therapy	1,516 ± 6,348	2,216 ± 8,177	0.135	1,925 ± 7,261	3,125 ± 10,366	0.156
Hormone therapy	305 ± 225	309 ± 244	0.725	299 ± 246	320 ± 285	0.752
Ovarian suppression[d]	1,628 ± 4,482	1,701 ± 4,490	0.398	716 ± 2,370	793 ± 2,587	0.221
Radiotherapy	285 ± 1,415	315 ± 1,541	0.915	389 ± 1,593	535 ± 2,162	0.659
Chemotherapy	33 ± 114	33 ± 121	0.225	33 ± 100	50 ± 152	0.893
Mental health or physiotherapy	322 ± 3,840	151 ± 965	0.839	872 ± 6,469	357 ± 1,368	0.682
Total costs	41,841 ± 16,384	9,102 ± 13,145	<0.001	42,792 ± 17,050	11,180 ± 15,281	<0.001
Total discounted costs	40,237 ± 15,392	8,510 ± 12,253	<0.001	41,137 ± 15,872	10,490 ± 14,337	<0.001

Continuous variables are summarized, with a mean ± SD. Significance of differences in sample characteristics between the arms were assessed with Fisher's exact test for binary, continuity-corrected chi-square test for categorical and Wilcoxon test for continuous parameters. [a]FU refers to the number of years between patient entry date and last reported outcome; [b]Missing describes the average fraction of patient-year records missing per patient within the 6-year FU period (includes both attrition and missing due to administrative censoring); [c]Excluding visits related to administration of ovarian suppression, including examinations by physicians, referral, and follow-up visits related to screenings and hospitalizations; [d]Includes goserelin or other luteinizing hormone-releasing hormone (LHRH) injections and not surgery or radiotherapy that are captured under the respective event types.
ET, endocrine therapy; FU, follow-up; PAL, palbociclib; QALYs, quality-adjusted life years.

TABLE 3 Cumulative incremental QALYs, costs, and cost-effectiveness ratios by year of FU without and with regression adjustment.

FU, years	Incremental QALYs, *n*	*p*-value	Incremental costs, EUR	*p*-value	ICER, EUR
Unadjusted					
1	0.003 (−0.011; 0.017)	0.668	31,422 (30,632, 32,211)	<0.001	10,248,892
1-2	0.021 (−0.010; 0.053)	0.178	32,884 (31,817; 33,950)	<0.001	1,529,645
1-3	0.064 (0.003; 0.124)	0.040	32,995 (31,606; 34,384)	<0.001	517,925
1-4	0.160 (0.041; 0.280)	0.009	33,636 (31,892; 35,380)	<0.001	209,934
Regression-adjusted					
1	0.000 (−0.012; 0.013)	0.959	31,441 (30,658; 32,224)	<0.001	93,371,819
1-2	0.013 (−0.019; 0.045)	0.437	32,863 (31,799; 33,926)	<0.001	2,579,213
1-3	0.049 (−0.008; 0.107)	0.094	32,865 (31,490; 34,239)	<0.001	667,611
1-4	0.088 (−0.001; 0.177)	0.054	33,336 (31,640; 35,033)	<0.001	380,001

The table shows mean and 95% confidence interval for cumulative incremental impacts of PAL on QALYs and costs over the respective years of FU. The unadjusted estimates were obtained by summing the incremental mean differences between FU years. Regression-adjusted estimates were obtained by summing the average marginal effects of PAL predicted for each year of FU from mixed-level linear models estimated on the full study population including data from all countries; see text for details. Data were censored to include patients who were present or dead at the end of each yearly FU. The total number of patient-year records used in the estimation was 2,987 for QALYs and 3,576 for costs. Unadjusted and regression-adjusted incremental impacts by arm and year of FU are reported in Tables A15 and A16 and mean totals by year in Figure A7 in Appendix A3.
FU, follow-up; ICER, incremental cost-effectiveness ratio; QALYs, quality-adjusted life years.

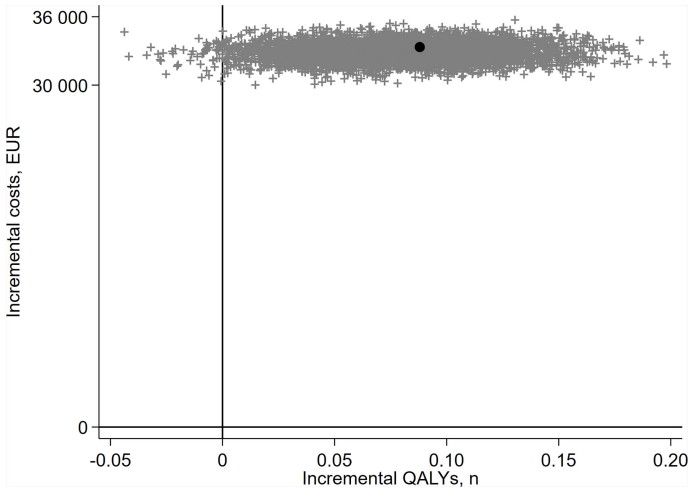


FIGURE 1
Bootstrap of regression-adjusted cumulative incremental outcomes plotted on the cost-effectiveness plane, 4 years of FU. Each dot represents a bootstrap replication (out of 5,000) of the cumulative incremental outcomes based on regression-adjusted results estimated at 4 years of FU. The black dot corresponds to the mean incremental QALYs of 0.088 and incremental costs of EUR 33,336 as reported in Table 4 above.

16)] and fairly closely clustered around the main result. The highest incremental gains, favoring PAL, were estimated in patients who received at least 80% of PAL doses, patients over 50, patients with ypN equal to 2 or 3, and patients with a risk score ≥ 3 (scenarios 3, 8, 6, and 12). Moreover, strong positive effects of PAL were estimated when data from year 5, based on less than 9% of patients (those enrolled early enough to reach this FU time point and not censored), were included (scenario 15). The estimated incremental gains doubled between years 4 and 5, leading to a total of 0.201 QALYs gained and an ICER of EUR 167,905 over 5 years of FU. Estimates based on multiply imputed data were similarly marginally positive and quantitatively comparable to the base-case results when using MMRM (scenarios 17–19). The incremental costs ranged between EUR 31,178 and 35,974. The largest incremental costs were estimated in patients who received at least 80% of PAL doses, while the lowest—indicating greater cost savings from PAL—were in patients with Ki-67 > 15%. The ICERs were mostly above EUR 300,000 and moved in a predictable pattern with changes in effectiveness. The overall range across scenarios was between EUR 167,905 and 1,603,238 per QALY gained.

Discussion

Key findings

We analyzed HRQoL and resource use data from the PENELOPE-B trial to estimate from the perspective of the German health statutory insurance the incremental effectiveness, expressed in QALYs, and costs of PAL added to standard-of-care ET in women with early breast cancer and at a high risk of relapse. Our primary result, regression-adjusted for stratification, missing data, and any residual imbalances at randomization, showed positive and marginally significant impacts of PAL on HRQoL at 4 years of FU. These impacts did not translate to differential care-seeking or cost savings, with nearly the full cost of PAL passed on to the system. The ICER was estimated at about EUR 380,000 per QALY gained, implying that PAL was not cost-effective compared to the standard-of-care ET at conventional willingness-to-pay thresholds.

We additionally observed that the effects of PAL on QALYs increased over time. We estimated relatively large and significant QALY gains in year 5, which led to an overall positive and significant cumulative effect over 5 years. This result should be interpreted with caution since few patients were followed this long. While PAL remained not cost-effective, more mature data on the effects of PAL beyond year 4, from PENELOPE-B or other trials, would be required to clarify the longer-term effects and, potentially, also the health-economic properties of PAL. We estimated numerically greater effects when averaged over all participating countries compared to Germany, suggesting that PAL+ET might be more effective in some settings. The differences in the magnitude and statistical significance compared to the full population were consistent with differences in patient characteristics and loss in power due to reduced sample size.

TABLE 4 Scenario analyses: regression-adjusted, 4 years of FU.

No	Rationale	Scenario	Incremental QALYs, n	*p*-value	Incremental costs, EUR	*p*-value	ICER
0		Base case	0.088 (−0.001; 0.177)	0.054	33,336 (31,640; 35,033)	<0.001	378,818
1	Heterogeneity	Patients recruited in Germany	0.021 (−0.141; 0.184)	0.797	33,668 (30,308; 37,028)	<0.001	1,603,238
2	Population	Per protocol population*	0.097 (0.007; 0.186)	0.035	33,381 (31,618; 35,144)	<0.001	344,134
3	Population	Patients randomized and treated	0.086 (−0.003; 0.174)	0.058	33,904 (32,240; 35,568)	<0.001	394,233
4	Population	Patients who received 80% of PAL doses	0.174 (0.087; 0.261)	<0.001	35,974 (34,392; 37,556)	<0.001	206,747
5	Risk factor	Patients with ypN 0-1	0.084 (−0.035; 0.203)	0.167	32,542 (30,203; 34,881)	<0.001	387,405
6	Risk factor	Patients with ypN 2-3	0.104 (−0.029; 0.237)	0.126	34,071 (31,521; 36,620)	<0.001	327,606
7	Risk factor	Age ≤ 50 years	0.034 (−0.086; 0.155)	0.579	34,672 (32,267; 37,076)	<0.001	1,019,765
8	Risk factor	Age > 50 years	0.157 (0.024; 0.289)	0.020	31,822 (29,468; 34,176)	<0.001	202,688
9	Risk factor	Patients with Ki-67 ≤ 15%	0.088 (−0.003; 0.179)	0.059	33,873 (31,839; 35,907)	<0.001	384,920
10	Risk factor	Patients with Ki-67 > 15%	0.078 (−0.134; 0.290)	0.472	31,335 (28,061; 34,10)	<0.001	401,731
11	Risk factor	Patients with CPS-EG score 2 and ypN+	0.062 (−0.065; 0.188)	0.341	33,415 (30,533; 36,297)	<0.001	538,952
12	Risk factor	Patients with CPS-EG score ≥ 3	0.094 (−0.030; 0.219)	0.138	33,401 (31,266; 35,535)	<0.001	355,330
13	Data limitations	Excluded non-breast-cancer hospitalizations	0.088 (−0.001; 0.177)	0.054	33,178 (31,571; 34,786)	<0.001	377,023
14	Data limitations	Included imputed expenditure in the year of death	0.088 (−0.001; 0.177)	0.054	33,293 (31,591; 34,995)	<0.001	378,330
15	Data limitations	Include data through year 5	0.201 (0.069; 0.332)	0.003	33,749 (31,450; 36,048)	<0.001	167,905
16	Missing values	Complete case analysis	−0.027 (−0.079; 0.025)	0.311	34,672 (32,745; 36,598)	<0.001	Detrimental
17	Missing values	MICE, OLS	0.103 (0.015; 0.191)	0.022	33,287 (31,655; 34,919)	<0.001	323,175
18	Correlation between outcomes	MICE, SUR	0.096 (0.000; 0.192)	0.051	35,070 (31,502; 38,638)	<0.001	365,313
19	Skewed outcomes	MICE, GLM	0.103 (0.015; 0.190)	0.021	33,288 (31,669; 34,907)	<0.001	323,184

The table presents the estimated regression-adjusted mean (95% confidence interval) differences between the arms in QALYs gained and costs incurred at 4 years of FU. The estimates were obtained by summing the average marginal effects of PAL predicted for each year of FU from mixed-level linear models; see text for details. Unless stated otherwise, data on QALYs and costs were censored to only include patients who were present or dead at the end of each yearly FU; item-missingness was relatively few and filled according to the algorithms detailed in the text. * See Loibl et al. (2021) (16)for exclusion of patients from per-protocol analysis. Scenarios 16–19 entailed multiple imputation with chained equations; missing values were filled following predictive mean matching (radius, five patients). Further details on MICE are in Appendix A2. The full set of scenarios evaluated are reported in Table A17 in Appendix A4
FU, follow-up; ICER, incremental cost-effectiveness ratio; GLM, generalized linear model; OLS, ordinary least squares; PAL, palbociclib; SUR, seemingly unrelated regressions; QALYs, quality-adjusted life years.

Policy implications and significance

To date, the economic analyses of PAL and other CDKi primarily focused on advanced and metastatic patients (42–47); these studies relied on data from several CDKi trials (48–52) to extrapolate impacts of CDKi to lifetime horizons in cohorts of patients. Overall, these model-based analyses estimated the incremental effectiveness of PAL compared to ET (most often with letrozole) between 0.32 and 1.39 QALYs gained with resulting ICERs, sensitive to price assumptions, between USD 150,000 and 800,000 per QALY (42–47). To our knowledge, this is the first study that evaluated the health-economic properties of PAL in early breast cancer patients. Considered together with the evidence of no clinical benefit shown by Loibl et al. (16), our findings do not support adding PAL to the standard-of-care ET in the adjuvant setting in high-risk patients in Germany.

Limitations

The analysis is subject to several limitations that may have impacted our results. These primarily stem from difficulties of collecting comprehensive resource use data alongside a clinical trial (53). Data collection forms were designed to minimize patient burden leading to some challenges in interpreting and valuing resource use (see Supplementary Materials 1). While these issues may have affected the level of costs, potentially biasing our estimates downward (54), the impact on incremental costs was likely minor since both clinical events and care episodes were well-balanced between the trial arms. Moreover, the costs of the care episodes most affected were relatively small compared to the initial costs of PAL treatment. These considerations were further supported with scenario analyses (Table A17 in Appendix A4, scenarios 13–23).

We carefully considered different sources of missing values in our data and applied appropriate strategies for valid inference. First, administrative censoring did not introduce bias but limited power to infer the impact of PAL over the maximum trial FU. Item-level missingness, to the extent that we could identify it, was relatively limited, leading us to adopt some *ad-hoc* solutions. We interpolated between FU points to fill in missing utility values and borrowed information from unaffected patients on types and quantities of resources used, which might have introduced some bias. The bigger challenge was dealing with attrition, which increased over the trial FU for both QALYs and costs. Our modeling strategy—MMRM—has been shown to be valid under MAR provided the random-effects structure was correctly specified (38). We used multiple imputation to allow for a more flexible correlation structure in patient random effects over time (38, 39). The estimated effects and their significance aligned well between the two methods.

Finally, we opted to model the outcomes with linear models despite both QALYs and costs being highly (left- and right-) skewed, yielding biased estimates of the mean. The main advantage of this functional form is the ease of interpretation; i.e., the estimated coefficients are directly interpretable as incremental impacts and can also be directly compared to descriptive means. Specifications using GLM with gamma log-link family following MICE that appropriately captured skewness in the data produced estimates that were nearly identical to our main specification (equal for impact on QALYs and higher for the costs with wider confidence intervals), suggesting that the linear models adequately captured the incremental differences between the arms.

Data availability statement

All relevant data are within this paper and its supporting information files. The data underlying the clinical results presented in the study are available from the German Breast Group. Some restrictions apply because of the confidentiality of patient data. Since these data are derived from a prospective clinical trial with ongoing follow-up collection, there are legal and ethical restrictions to sharing sensitive patient-related data publicly. Interested groups may use the Cooperation Proposal Form at https://www.gbg.de/en/research/trafo.php. Data can be requested in the context of a translational research project by sending the form to trafo@gbg.de. Translational research proposals are approved by the GBG scientific boards.

Ethics statement

This study was reviewed and approved by Country ethics committees or institutional review boards. The patients/participants provided their written informed consent to participate in this study.

Author contributions

KG developed the analytical methodology, sourced unit costs and other inputs for the calculation of QALYs and costs, conducted the analysis, and wrote the first draft. SL acquired funding, designed the clinical study, and led data collection and analysis of clinical outcomes. PS sourced unit costs and other inputs for the calculation of QALYs and costs, and supported the analysis. FM, MM, MU, HRB, S-BK, HDB, NM, KAG, JG-S, CK, TR, MT, HR, MG, and AM collected the data. NB led the analysis of clinical outcomes. MS acquired funding, designed the health-economic study, supported the analysis, and revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the German Breast Group, which received funding from Pfizer. Pfizer supported the conduct of the trial financially and provided the drug. Pfizer was not involved in the design of the health-economic study, collection, analysis, or interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Acknowledgments

We thank Daniel Droeschel for providing an overview of market reimbursement and healthcare financing within the German healthcare system and helping source unit costs, Sabine Seiler and Jenny Furlanetto for providing expert guidance on clinical management and patient pathways in early breast cancer, Christiane Praetor for clarifying data collection and data processing within PENELOPE-B trial and related documentation, and Michaela Barbier and Judith Lupatch for helpful discussions on methods for dealing with missing data in randomized controlled trials. The contributors are not in any way responsible for the content of this manuscript. The responsibility for the content is with the authors.

Conflict of interest

KG received research funding from Novartis paid to the institution. SL received research funding from Abbvie, AstraZeneca, Celgene, Daiichi Sankyo, Immunomedics/Gilead, Novartis, and Pfizer; royalties (Digital Ki67 Evaluator) and honorarium from Abbvie, Amgen, Bayer, BMS, Celgene,

Eirgenix, GSK, Lilly, Merck, AstraZeneca, Pierre Fabre, Prime/Medscape, Daiicho Sankyo, Novartis, Pfizer, Immunomedics/Gilead, Puma, Seagen, and Samsung; and reported patents (EP14153692.0, EP21152186.9, EP15702464.7, EP19808852.8, Digital Ki67 Evaluator) all paid to the institution. PS received research funding from Novartis paid to the institution. FM received research funding from Roche, Novartis, AstraZeneca, GSK/Tesaro, MED, Clovis, Vaccibody, Gilead Sciences, and Eisai, and consulting fees from Vacibody all paid to the institution; received personal consulting fees and honorarium from AstraZeneca, Clovis, GSK/Tesaro, Eli Lilly, Novartis, Pfizer, Roche, Eisai, GenomicHealth, Myriad Genetics, PharmaMar, MSD, Immunomedis/Gilead, Pierre-Fabre, AGENDIA, and Seattle Genetics, and support for meetings and travel from Pfizer, Roche, and AstraZeneca; and participated in Advisory Boards for Palles and Amgen. MU received consulting fees from Abbvie, Astra Zeneca, Amgen, Celgene, Daiichi Sankyo, Eisai, Gilead, Lilly, Molecular Health, MSD Merck, Mylan, Novartis, Pierre Fabre, Pfizer, Roche, and Seagen, and honorarium from Amgen, Astra Zeneca, BMS, Celgene, Daiichi Sankyo, Gilead, GSK, Lilly, Mundipharma, Novartis, Pierre Fabre, Pfizer, Roche, Sanofi Aventis, and Seagen all paid to the institution. HRB received personal consulting fees from AstraZeneca, and received support for attending meetings and travel from Pfizer, Daiichi Sankyo, and Roche. S-BK received research funding from Novartis, Sanofi-Aventis, and DongKook paid to the institution; received personal consulting fees and honorarium from Novartis, AstraZeneca, Eli Lilly, Dae Hwa, ISU Abxis, Daiichi Sankyo, and BeiGene; participated in Advisory Boards for Novartis, AstraZeneca, Eli Lilly, Dae Hwa, ISU Abxis, Daiichi Sankyo, and BeiGene; serves as a Co-Chair for ESMO Breast 2021-2022; and holds stock of Genopeaks and Neogene TC. HDB received research funding from NSABP to the institution and reported stock ownership in Pfizer. NM participated in an Advisory Board for Pfizer. KAG received research funding from Pfizer, AstraZeneca, Eli Lilly, Roche, Merck, Gilead, Novartis, Ayala, BMS, and Seagen paid to the institution; received personal consulting fees and honorarium from AstraZeneca, Eli Lilly, Pfizer, Merck, Novartis, Gilead, Seagen, and GenomicHealth; received payment from expert testimony from Genetech; and participated in Advisory Boards for Ayala and AstraZeneca. JG-S received personal consulting fees and honorarium from AstraZeneca, Gilead, Novartis, Daiichi Sankyo, Eli Lilly, Exact Sciences, Seagen, Sanofi, EISAI, MSD, and Celgene. CK received research funding from Health Research Board and Mater Foundation paid to the institution; received personal consulting fees and honorarium from Exact Sciences and Daiichi Sankyo; received support for attending meetings and travel from Daiichi Sankyo and Roche; and participated in Advisory Board for Daiichi Sankyo. TR received personal consulting fees and honorarium from Pfizer. MT received research funding from Chugai, Takeda, Pfizer, Kyowa-Kirin, Taiho, JBCRG assoc., KBCRN assoc., Eisai, Eli Lilly, Daiichi Sankyo, AstraZeneca, Astellas, Shimadzu, Yakult, Nippon Kayaku, AFI technology, Luxonus, Shionogi, and GL Science paid to the institution; received honorarium from Chugai, Takeda, Pfizer, Kyowa-Kirin, Taiho, Eisai, Daiichi Sankyo, AstraZeneca, Eli Lilly, MSD, Exact Science, Novartis, Shimadzu, Yakult, Nippon Kayaku, and Devicore Medical Japan; and participated in Advisory Boards for Kyowa-Kirin, Daiichi Sankyo, Eli Lilly, BMS, Athenex Oncology, Bertis, Terumo, and Kansai Medical Net. HSR received research funding (study materials only) from Pfizer, Merck, Novartis, Eli Lilly, Roche, Daiichi Sankyo, Seattle Genetics, Macrogenics, Sermonix, Boehringer Ingelheim, Polyphor, AstraZeneca, Ayala, and Gilead paid to the institution; and received personal consulting fees and honorarium from Napo, Puma, Mylan, and Samsung. MG received personal consulting fees and honorarium from AstraZeneca, Eli Lilly, Daiichi Sankyo, Amgen, Veracyte, Novartis, Pierre Fabre, MSD, and Life Brain; received payment for expert testimony from Eli Lilly; and reported an immediate family member employed by Sandoz. AM received personal consulting fees and honoraria from Pfizer. MS received research funding from AbbVie, Biogen, Bristol Myers Squibb, Merck Sharpe and Dohme, Mundipharma, Novartis, and Roche paid to the institution; and received personal consulting fees from BMS and Sandoz.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.886831/full#supplementary-material

References

1. European Commission. *ECIS - European cancer information system* (2021). Available at: https://ecis.jrc.ec.europa.eu/ (Accessed May 15, 2021).

2. OECD/European Union. Breast cancer stage distribution, women diagnosed during 2010-14, in: *Health at a glance: Europe 2020: State of health in the EU cycle* (2020). Paris: OECD Publishing. Available at: https://doi.org/10.1787/609adf54-en (Accessed May 15, 2021).

3. O'Shaughnessy J. Extending survival with chemotherapy in metastatic breast cancer. *oncol* (2005) 10:20–9. doi: 10.1634/theoncologist.10-90003-20

4. Waks AG, Winer EP. Breast cancer treatment: A review. *JAMA* (2019) 321 (3):288–300. doi: 10.1001/jama.2018.19323

5. Thomssen C, Balic M, Harbeck N, Gnant M. St. Gallen/Vienna 2021: a brief summary of the consensus discussion on customizing therapies for women with early breast cancer. *Breast Care* (2021) 16(2):135–43. doi: 10.1159/000516114

6. Li J, Huo X, Zhao F, Ren D, Ahmad R, Yuan X, et al. Association of cyclin-dependent kinases 4 and 6 inhibitors with survival in patients with hormone receptor-positive metastatic breast cancer: A systematic review and meta-analysis. *JAMA Netw Open* (2020) 3(10):e2020312. doi: 10.1001/jamanetworkopen.2020.20312

7. Martin JM, Goldstein LJ. In support of CDK4/6 inhibitors-a meta-analysis of available randomized data. *JAMA Netw Open* (2020) 3(10):e2021062. doi: 10.1001/jamanetworkopen.2020.21062

8. Spring LM, Wander SA, Andre F, Moy B, Turner NC, Bardia A. Cyclin-dependent kinase 4 and 6 inhibitors for hormone receptor-positive breast cancer: past, present, and future. *Lancet* (2020) 395(10226):817–27. doi: 10.1016/S0140-6736(20)30165-3

9. Finn RS, Dering J, Conklin D, Kalous O, Cohen DJ, Desai AJ, et al. PD 0332991, a selective cyclin d kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. *Breast Cancer Res* (2009) 11(5):1–13. doi: 10.1186/bcr2419

10. Fry DW, Harvey PJ, Keller PR, Elliott WL, Meade M, Trachet E, et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Mol Cancer Ther* (2004) 3 (11):1427–38. doi: 10.1158/1535-7163.1427.3.11

11. Serra F, Lapidari P, Quaquarini E, Tagliaferri B, Sottotetti F, Palumbo R. Palbociclib in metastatic breast cancer: current evidence and real-life data. *Drugs Context* (2019) 8:212579. doi: 10.7573/dic.212579

12. US Food & Drug Administration. *Palbociclib (IBRANCE capsules)* (2015). Available at: https://www.fda.gov/drugs/resources-information-approved-drugs/palbociclib-ibrance-capsules (Accessed May 15, 2021).

13. EMA. *New treatment for breast cancer* (2016). Available at: https://www.ema.europa.eu/en/news/new-treatment-breast-cancer (Accessed May 15, 2021).

14. Cunningham NC, Turner NC. Understanding divergent trial results of adjuvant CDK4/6 inhibitors for early stage breast cancer. *Cancer Cell* (2021) 39 (3):307–9. doi: 10.1016/j.ccell.2021.02.011

15. Mayer EL, Dueck AC, Martin M, Rubovszky G, Burstein HJ, Bellet-Ezquerra M, et al. Palbociclib with adjuvant endocrine therapy in early breast cancer (PALLAS): interim analysis of a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* (2021) 22(2):212–22. doi: 10.1016/S1470-2045(20)30642-2

16. Loibl S, Marme F, Martin M, Untch M, Bonnefoi H, Kim SB, et al. Palbociclib for residual high-risk invasive HR-positive and HER2-negative early breast cancer-the Penelope-b trial. *J Clin Oncol* (2021) 39(14):1518–30. doi: 10.1200/JCO.20.03639

17. Gnant M, Dueck AC, Frantal S, Martin M, Burstein HJ, Greil R, et al. Adjuvant palbociclib for early breast cancer: The PALLAS trial results (ABCSG-42/AFT-05/BIG-14-03). *J Clin Oncol* (2021) 40(3):282–93. doi: 10.1200/JCO.21.02554

18. Claxton K. The irrelevance of inference: a decision-making approach to the stochastic evaluation of health care technologies. *J Health Econ* (1999) 18(3):341–64. doi: 10.1016/S0167-6296(98)00039-3

19. Whitehurst DG, Bryan S. Trial-based clinical and economic analyses: the unhelpful quest for conformity. *Trials* (2013) 14:421. doi: 10.1186/1745-6215-14-421

20. CGM. *Lauer-taxe* (2020). Available at: https://www.cgm.com/deu_de/produkte/apotheke/lauer-taxe.html (Accessed May 15, 2020).

21. KBV. *Honorarbericht* (2020). Available at: https://www.kbv.de/html/honorarbericht.php (Accessed May 15, 2020).

22. KBV. *Einheitlicher bewertungsmaßstab (EBM), online-version des EBM* (2020). Available at: https://www.kbv.de/html/online-ebm.php (Accessed May 15, 2020).

23. Deutsche Rentenversicherung. *Reha-bericht* (2019). Available at: https://www.deutsche-rentenversicherung.de/SharedDocs/Downloads/DE/Statistiken-und-Berichte/Berichte/rehabericht_2019.html (Accessed May 15, 2020).

24. Arnold M, Pfeifer K, Quante AS. Is risk-stratified breast cancer screening economically efficient in Germany? *PloS One* (2019) 14(5):e0217213. doi: 10.1371/journal.pone.0217213

25. InEK. *aG-DRG-System 2020 fallpauschalen-katalog* (2020). Available at: https://www.g-drg.de/aG-DRG-System_2020/Fallpauschalen-Katalog/Fallpauschalen-Katalog_2020 (Accessed May 15, 2020).

26. Greiner W, Claes C, Busschbach JJ, von der Schulenburg JM. Validating the EQ-5D with time trade off for the German population. *Eur J Health Econ* (2005) 6 (2):124–30. doi: 10.1007/s10198-004-0264-z

27. Laird NM, Ware JH. Random-effects models for longitudinal data. *Biometrics* (1982) 38(4):963–74. doi: 10.2307/2529876

28. National Library of Medicine (US). *A study of palbociclib in addition to standard endocrine treatment in hormone receptor positive Her2 normal patients with residual disease after neoadjuvant chemotherapy and surgery (PENELOPE-b)* (2021). Available at: https://clinicaltrials.gov/ct2/show/NCT01864746 (Accessed December 12, 2021).

29. Mittendorf EA, Jeruss JS, Tucker SL, Kolli A, Newman LA, Gonzalez-Angulo AM, et al. Validation of a novel staging system for disease-specific survival in patients with breast cancer treated with neoadjuvant chemotherapy. *J Clin Oncol* (2011) 29(15):1956. doi: 10.1200/JCO.2010.31.8469

30. EuroQol Research Foundation. *EQ-5D-3L user guide* (2018). Available at: https://euroqol.org/publications/user-guides/ (Accessed December 12, 2021).

31. Group TE. EuroQol-a new facility for the measurement of health-related quality of life. *Health policy* (1990) 16(3):199–208. doi: 10.1016/0168-8510(90)90421-9

32. Manca A, Hawkins N, Sculpher MJ. Estimating mean QALYs in trial-based cost-effectiveness analysis: the importance of controlling for baseline utility. *Health econ.* (2005) 14(5):487–96. doi: 10.1002/hec.944

33. FRB. *FRED economic data, GDP deflator* (2020). Available at: https://fred.stlouisfed.org/series/GDPDEF (Accessed December 12, 2021).

34. Cook JR, Drummond M, Glick H, Heyse JF. Assessing the appropriateness of combining economic data from multinational clinical trials. *Stat Med* (2003) 22 (12):1955–76. doi: 10.1002/sim.1389

35. Ramsey SD, Willke RJ, Glick H, Reed SD, Augustovski F, Jonsson B, et al. Cost-effectiveness analysis alongside clinical trials II-an ISPOR good research practices task force report. *Value Health* (2015) 18(2):161–72. doi: 10.1016/j.jval.2015.02.001

36. Gail M, Simon R. Testing for qualitative interactions between treatment effects and patient subsets. *Biometrics* (1985) 41(2):361–72. doi: 10.2307/2530862

37. Kahan BC, Morris TP. Improper analysis of trials randomised using stratified blocks or minimisation. *Stat Med* (2012) 31(4):328–40. doi: 10.1002/sim.4431

38. DeSouza CM, Legedza AT, Sankoh AJ. An overview of practical approaches for handling missing data in clinical trials. *J Biopharm Stat* (2009) 19(6):1055–73. doi: 10.1080/10543400903242795

39. Jakobsen JC, Gluud C, Wetterslev J, Winkel P. When and how should multiple imputation be used for handling missing data in randomised clinical trials–a practical guide with flowcharts. *BMC Med Res methodol.* (2017) 17(1):1–10. doi: 10.1186/s12874-017-0442-1

40. Faria R, Gomes M, Epstein D, White IR. A guide to handling missing data in cost-effectiveness analysis conducted within randomised controlled trials. *Pharmacoeconomics* (2014) 32(12):1157–70. doi: 10.1007/s40273-014-0193-3

41. StataCorp. *Stata statistical software: Release 16*. College Station, TX: StataCorp LP (2019).

42. Jeong E, Wang C, Wilson L, Zhong L. Cost-effectiveness of adding ribociclib to endocrine therapy for patients with HR-positive, HER2-negative advanced breast cancer among premenopausal or perimenopausal women. *Front Oncol* (2021) 11:981. doi: 10.3389/fonc.2021.658054

43. Zhang B, Long EF. Cost-effectiveness analysis of palbociclib or ribociclib in the treatment of advanced hormone receptor-positive, HER2-negative breast cancer. *Breast Cancer Res Treat* (2019) 175(3):775–9. doi: 10.1007/s10549-019-05190-3

44. Zhang Y, Zeng X, Deng H, Ma F, Peng Y, Yi L, et al. Cost-effectiveness analysis of adding palbociclib as a second-line endocrine therapy for HR(+)/HER2(-) metastatic breast cancer from the US and Chinese perspectives. *Clin Ther* (2019) 41(6):1175–85. doi: 10.1016/j.clinthera.2019.04.033

45. Raphael J, Helou J, Pritchard K, Naimark D. Palbociclib in hormone receptor positive advanced breast cancer: a cost-utility analysis. *Eur J Cancer* (2017) 85:146–54. doi: 10.1016/j.ejca.2017.08.018

46. Matter-Walstra K, Schwenkglenks M, Dedes KJ. Cost-effectiveness of palbociclib plus letrozole versus letrozole alone as a first-line treatment in women with oestrogen receptor-positive, HER2-negative, advanced breast cancer. revised results for the Swiss health care setting. *Breast Cancer Res Treat* (2017) 163(3):635. doi: 10.1007/s10549-017-4209-5

47. Jayasekera J, Mandelblatt JS. Systematic review of the cost effectiveness of breast cancer prevention, screening, and treatment interventions. *J Clin Oncol* (2020) 38(4):332. doi: 10.1200/JCO.19.01525

48. Cristofanilli M, Turner NC, Bondarenko I, Ro J, Im S-A, Masuda N, et al. Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *Lancet Oncol* (2016) 17(4):425–39. doi: 10.1016/S1470-2045(15)00613-0

49. Rossi V, Berchialla P, Giannarelli D, Nisticò C, Ferretti G, Gasparro S, et al. Should all patients with HR-positive HER2-negative metastatic breast cancer receive CDK 4/6 inhibitor as first-line based therapy? a network meta-analysis of data from the PALOMA 2, MONALEESA 2, MONALEESA 7, MONARCH 3, FALCON, SWOG and FACT trials. *Cancers* (2019) 11(11):1661. doi: 10.3390/cancers11111661

50. Johnston S, O'Shaughnessy J, Martin M, Huober J, Toi M, Sohn J, et al. Abemaciclib as initial therapy for advanced breast cancer: MONARCH 3 updated results in prognostic subgroups. *NPJ Breast Cancer* (2021) 7(1):1–5. doi: 10.1038/s41523-021-00289-7

51. Tripathy D, Im S-A, Colleoni M, Franke F, Bardia A, Harbeck N, et al. Ribociclib plus endocrine therapy for premenopausal women with hormone-receptor-positive, advanced breast cancer (MONALEESA-7): a randomised phase 3 trial. *Lancet Oncol* (2018) 19(7):904–15. doi: 10.1016/S1470-2045(18)30292-4

52. O'shaughnessy J, Petrakova K, Sonke GS, Conte P, Arteaga CL, Cameron DA, et al. Ribociclib plus letrozole versus letrozole alone in patients with *de novo* HR+, HER2– advanced breast cancer in the randomized MONALEESA-2 trial. *Breast Cancer Res Treat* (2018) 168(1):127–34. doi: 10.1007/s10549-017-4518-8

53. O'Sullivan AK, Thompson D, Drummond MF. Collection of health-economic data alongside clinical trials: is there a future for piggyback evaluations? *Value Health* (2005) 8(1):67–79. doi: 10.1111/j.1524-4733.2005.03065.x

54. Kreis K, Plothner M, Schmidt T, Seufert R, Schreeb K, Jahndel V, et al. Healthcare costs associated with breast cancer in Germany: a claims data analysis. *Eur J Health Econ* (2020) 21(3):451–64. doi: 10.1007/s10198-019-01148-w

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 01 December 2022
DOI 10.3389/fonc.2022.1016295

OPEN ACCESS

EDITED BY
Ariella Hanker,
University of Texas Southwestern Medical Center, United States

REVIEWED BY
Elena Provenzano,
Cambridge Biomedical Research Centre (NIHR), United Kingdom
Sadaf Alipour,
Tehran University of Medical Sciences, Iran

*CORRESPONDENCE
Angela Toss
angela.toss@unimore.it

†These authors have contributed equally to this work and share first autorship

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 10 August 2022
ACCEPTED 09 November 2022
PUBLISHED 01 December 2022

CITATION
Toss A, Venturelli M, Civallero M, Piombino C, Domati F, Ficarra G, Combi F, Cabitza E, Caggia F, Barbieri E, Barbolini M, Moscetti L, Omarini C, Piacentini F, Tazzioli G, Dominici M and Cortesi L (2022) Predictive factors for relapse in triple-negative breast cancer patients without pathological complete response after neoadjuvant chemotherapy.
Front. Oncol. 12:1016295.
doi: 10.3389/fonc.2022.1016295

Predictive factors for relapse in triple-negative breast cancer patients without pathological complete response after neoadjuvant chemotherapy

Angela Toss[1,2*†], Marta Venturelli[1†], Monica Civallero[3], Claudia Piombino[1], Federica Domati[2], Guido Ficarra[4], Francesca Combi[5,6], Eleonora Cabitza[1], Federica Caggia[1], Elena Barbieri[1], Monica Barbolini[1], Luca Moscetti[1], Claudia Omarini[1], Federico Piacentini[1,2], Giovanni Tazzioli[2,5], Massimo Dominici[1,2] and Laura Cortesi[1]

[1]Department of Oncology and Hematology, Azienda Ospedaliero-Universitaria di Modena, Modena, Italy, [2]Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, Modena, Italy, [3]Department of Surgery, Medicine, Dentistry and Morphological Sciences with Transplant Surgery, Oncology and Regenerative Medicine Relevance, University of Modena and Reggio Emilia, Modena, Italy, [4]Pathology Unit, University Hospital of Modena, Modena, Italy, [5]Unit of Breast Surgical Oncology, Azienda Ospedaliero-Universitaria di Modena, Modena, Italy, [6]Department of Biomedical, Metabolic and Neural Sciences, International Doctorate School in Clinical and Experimental Medicine, University of Modena and Reggio Emilia, Modena, Italy

Introduction: Triple-negative breast cancer (TNBC) patients who do not obtain pathological complete response (pCR) after neoadjuvant chemotherapy (NACT) present higher rate of relapse and worse overall survival. Risk factors for relapse in this subset of patients are poorly characterized. This study aimed to identify the predictive factors for relapse in TNBC patients without pCR after NACT.

Methods: Women with TNBC treated with NACT from January 2008 to May 2020 at the Modena Cancer Center were included in the analysis. In patients without pCR, univariate and multivariable Cox analyses were used to determine factors predictive of relapse.

Results: We identified 142 patients with a median follow-up of 55 months. After NACT, 62 patients obtained pCR (43.9%). Young age at diagnosis (<50 years) and high Ki-67 (20%) were signi!cantly associated with pCR. Lack of pCR after NACT resulted in worse 5-year event-free survival (EFS) and overall survival (OS). Factors independently predicting EFS in patients without pCR were the presence of multifocal disease [hazard ratio (HR), 3.77; 95% CI, 1.45–9.61; p=0.005] and residual cancer burden (RCB) III (HR, 3.04; 95% CI, 1.09–9.9; p=0.04). Neither germline BRCA status nor HER2-low expression were associated with relapse.

Discussion: These data can be used to stratify patients and potentially guide treatment decision-making, identifying appropriate candidates for treatment intensi!cation especially in neo-/adjuvant setting.

KEYWORDS

triple-negative breast cancer, neoadjuvant chemotherapy, pathologic response, residual cancer burden (RCB), multifocal disease

Introduction

Triple-negative breast cancer (TNBC) is defined by the lack of estrogen receptor (ER) and progesterone receptor (PR) expression and HER2 gene amplification. TNBCs present aggressive biology, with higher risk of local and distant recurrence compared to other subtypes, rapid progression with short response duration to therapies, and poor survival outcomes. They are typically diagnosed at younger age (1) and are more likely to present as a palpable mass becoming clinically apparent between annual screening mammograms ("interval cancer") (2). At diagnosis, the majority of TNBC patients present with stage T2 or T3 and have involved lymph nodes and positive lymphovascular invasion (3). Metastatic diseases are more likely to occur in the viscera and brain compared to other breast cancer subtypes with a lower prevalence of bone metastasis (1). Moreover, most of the metastatic disease occurs within 2 or 3 years from diagnosis (1), whereas women who do not relapse during this time have similar survival rates of hormone receptor (HR)-positive BC.

Chemotherapy is the backbone for patients with TNBC in neoadjuvant (NACT), adjuvant, and metastatic setting, and despite its aggressive behavior, TNBC is particularly sensitive to cytotoxic chemotherapy ("triple negative paradox"). In the early stage, it is well established that the long-term outcome of neoadjuvant or adjuvant chemotherapy approach is the same. Nonetheless, NACT, initially used only to convert unresectable tumors into resectable ones, reduces the extent of surgery and improves cosmetic outcomes. Furthermore, the occurrence of a pathological complete response (pCR) after NACT emerged as an indicator of responsiveness to standard therapy. Indeed, patients who obtain pCR—defined as a lack of invasive disease in both breast and lymph nodes—showed improved outcomes in terms of event-free survival (EFS) and OS, whereas residual disease post-NACT is predictive of early recurrences and mortality (4, 5). For all these reasons, NACT became the standard of treatment also for patients with operable disease. However, most patients treated with standard anthracycline- and taxane-based NACT do not experience pCR (5, 6), and data are scarce regarding factors predictive of relapse in this subgroup of patients with residual disease after NACT. The present study aimed to identify factors predictive of relapse in patients with TNBC without pCR after NACT.

Materials and methods

Study population and design

A retrospective review of the electronic medical records of the Unit of Breast Surgery was performed, and 142 patients treated with NACT for early or locally advanced TNBC between January 2008 and May 2020 at the University Hospital of Modena were identified. All the patients with clinical data available, age ≥8 years, and diagnosed with early/locally advanced TNBC (T1–4, N0/+) who underwent NACT were included in the study. Exclusion criteria were diagnosis of hormone-receptor-positive and/or HER2-positive tumors, stage IV cancer, and primary surgery followed by adjuvant chemotherapy.

Tumor-specific characteristics, including tumor and nodal stage, histology, grade, and lymphovascular space invasion, were collected. Furthermore, patient, radiological, and treatment features were evaluated, including age at diagnosis and body mass index (BMI). In particular, data about breast MRI, cytohistological examination from fine-needle aspiration (FNA)/core biopsy, and multicentricity/multifocality or bilaterality were collected. Cytohistological examinations were determined by a pathologist at the time of surgery. Multicentricity/multifocality was defined as more than one foci of tumor in the same breast at the radiological examination before the surgery, independent of quadrant or distance.

The type of NACT, either within clinical trials or based on standard guidelines, and the type of breast and axillary surgery and adjuvant treatments were analyzed. Data about pathological downstaging and pCR, defined as no evidence of invasive disease in the breast and lymph nodes (ypT0/is, ypN0), were collected. In patients not achieving pCR, residual cancer burden (RCB) (7) and tumor-infiltrating lymphocytes (TILs) (8) were assessed. Time from NACT end to surgery and time from surgery to radiation therapy were included in this analysis. Finally, patients' data concerning germline mutational status of *BRCA1* or *BRCA2* or about other breast/ovarian hereditary cancer syndrome genes, when available, were included as well.

This retrospective monocentric study was approved by our local Ethical Committee of Area Vasta Emilia Nord (Prot. AOU 25084/20).

Statistical analysis and outcome measures

Baseline differences for clinical and demographic endpoints between patients with and without pCR were assessed by chi-square test or Fisher's exact test (two-sided) for data analysis. A p-value < 0.05 was considered statistically significant. Outcomes of interest were event-free survival (EFS) and OS, and survival estimates were calculated and reported at 5 years. Time intervals were calculated from diagnosis until death or last follow-up. Patients were censored at the date of last clinical contact. EFS was defined as the time from the date of the diagnosis to the date of the first documented relapse (local, regional, and/or distant), while OS was defined as the time from diagnosis of BC to death/last follow-up. Overall survival and presence/absence of relapse was compared between patients with and without pCR after NACT using the Kaplan–Meier method. Survival estimates were calculated and reported at 5 years, along with their 95% confidence intervals (95% CIs), in the pCR and no pCR group. In patients without pCR, univariate and multivariable Cox analyses were used to determine factors predictive of relapse. Statistical analyses were done using IBM SPSS Statistics for Windows Version 23.0 (IBM Corporation, Armonk, NY, USA).

Results

Patients and treatment characteristics

A total of 142 patients were identified and included in this study. Median follow-up was 55 months (range, 7–155 months). The characteristics of TNBC patients are listed in Table 1. BMI at diagnosis was considered normal (range, 18.5–24.9) in 62.8% of patients. Of the patients, 73.2% underwent genetic testing, and 30% of them presented germline likely pathogenic or pathogenic variants in cancer predisposition genes (24 BRCA1, 4 BRCA2, 1 PALB2, 1 NBN, and 1 ATM). Most of the patients presented clinical tumor stage cT2 (75.2%) and no lymph nodes involvement (56%). TNBCs were predominantly monolateral (97.2%) and unifocal (71.8%).

Treatment characteristic, response to NACT, and histopathological tumor characteristic on the surgical tissue are shown in Table 2. A total of 109 patients (76.8%) underwent an anthracycline and taxane-based NACT, 6 patients (4.2%) underwent an anthracycline or taxane-based NACT, whereas 27 patients (19.0%) underwent platinum-based NACT. Overall, 60.6% had pre-treatment breast MRI. The average time between neoadjuvant chemotherapy and surgery was 31.4 days (range, 16–74). Seventy-four patients (53.6%) had mastectomy, 83 (58.9%) had sentinel lymph node biopsy (SLNB) performed, and 68 patients (48.6%) had at least 11 lymph nodes removed at the definitive surgery. pCR was obtained in 62 patients (43.9%). After NACT, most of the patients

TABLE 1 Characteristics of TNBC patients and histopathological tumor characteristics on tumor biopsy.

Characteristic	N(%)
Total	142
Age at diagnosis	
<50	68 (47.9%)
≥50	74 (52.1%)
Unknown	0
Genetic mutation	
No	73 (70.2%)
Yes	31 (29.8%)
Unknown	38
BMI	
<25	86 (62.8%)
≥25	51 (37.2%)
Unknown	5
Bilateral disease	
No	138 (97.2%)
Yes	4 (2.8%)
Unknown	0
Multifocal disease	
No	102 (71.8%)
Yes	40 (28.2%)
Unknown	0
Histology on tumor biopsy	
Ductal	131 (93.6%)
Other	9 (6.4%)
Unknown	2
Grade on tumor biopsy	
II	4 (3.3%)
III	119 (96.7%)
Unknown	19
Ki-67 on tumor biopsy	
<20	10 (7.1%)
≥20	132 (92.9%)
Unknown	0
HER2 on tumor biopsy	
0	57 (41.0%)
1+/2+(ISH negative)	82 (58.9%)
Unknown	3
Clinical T stage	
T1	19 (13.5%)
T2	106 (75.2%)
T3	6 (4.2%)
T4	10 (7.1%)
Unknown	1
Clinical N stage	
N0	80 (56.3%)
N+	62 (43.7%)
Unknown	0

10.3389/fonc.2022.1016295

TABLE 2 Treatment characteristics, response to neoadjuvant chemotherapy, and histopathological tumor characteristic on surgical tissue.

Characteristic	N (%)
Total	142
MRI	
No	56 (39.4%)
Yes	86 (60.6%)
Unknown	0
Time NACT-Surgery	Median 31.4 days (16–74)
<30 days	63
≥30 days	74
Type surgery	
Conservative	64 (46.4%)
Mastectomy	74 (53.6%)
Unknown	4
SLNB	
No	58 (41.1%)
Yes	83 (58.9%)
Unknown	1
Type of NACT	
Anthracycline and taxane-based	109 (76.8%)
Anthracycline or taxane-based	6 (4.2%)
Platinum-based	27 (19.0%)
Unknown	0
ypT	
0	67 (47.9%)
1	73 (52.1%)
Unknown	2
ypN	
0	117 (82.9%)
1	24 (17.1%)
Unknown	1
LN assessment	
≤10	72 (51.4%)
>10	68 (48.6%)
Unknown	2
pCR	
No	79 (56.1%)
Yes	62 (43.9%)
Unknown	1
Residual cancer burden	
I	3 (3.9%)
II	59 (77.6%)
III	14 (18.4%)
Unknown	3
TILs on residual tumor	
<30%	39 (51.3%)
≥30%	37 (48.7%)
Unknown	3
Grading on residual tumor	
II	4 (6.2%)
III	61 (93.8%)
Unknown	14
Ki-67 on residual tumor	
<20	27 (37.5%)
≥20	45 (62.5%)
Unknown	7
Ki67 pre- *vs.* post-NACT	
Stable/increased	19 (27.1%)
Decreased	51 (72.9%)
Unknown	9
LVSI on residual tumor	
No	35 (72.9%)
Yes	13 (27.1%)
Unknown	31
Adjuvant treatment	
No	112 (79.4%)
Yes	29 (20.6%)
Unknown	1
Time surgery-RT	Median, 84.6 days (43–171)
≤90 days	44
>90 days	21

MRI, magnetic resonance imaging; NACT, neoadjuvant chemotherapy; SLNB, sentinel lymph node biopsy; LN, lymph node; pCR, pathological complete response; LVSI, lymphovascular space invasion; RT, radiotherapy.

had high nuclear-grade residual (93.8%), high Ki-67 index (62.5%), and no lymphovascular space invasion (LVSI) (72.9%). In 72.9% of cases, Ki-67 index decreased after NACT.

Adjuvant treatment was prescribed in 29 patients (20.6%) (capecitabine in 7 cases, immunotherapy in 6, capecitabine and immunotherapy in 1 case, olaparib in 3, and other chemotherapy agents in 11 patients), while adjuvant radiotherapy was performed in 86 patients (60.9%). The average time between surgery and radiotherapy was 84.6 days (range, 43–171).

Outcomes

Seven out of 62 patients (9.7%) who achieved pCR relapsed (three patients with loco-regional disease and four with distant metastasis). Among the 80 patients who did not achieve pCR, one was lost to follow-up, and information regarding her tumor residual and outcome are is available. On the other hand, 23 out of 79 patients (29.1%) who did not achieve pCR relapsed. Of those, 12 patients had loco-regional relapse, and 11 had distant disease. As shown in Figure 1, patients who obtained pCR after NACT showed better EFS (5-year EFS 90% *vs.* 70%, p=0.008) and OS (5-year OS, 95% *vs.* 69%, p=0.003). Overall, young age at diagnosis (<50 years) and high Ki-67 (≥ 20%) were significantly

associated with pCR (Supplementary Table S1). Moreover, although not statistically significant, pCR rate was higher with platinum-based NACT (51.8%) than without platinum agents (42.1%).

Univariate and multivariable analyses

In univariate analysis, factors associated with relapse in the cohort of patients with residual disease after NACT (79 patients) were BMI > 25, bilateral BC, multifocal disease, clinical T3–T4 stage, clinical N+ stage, RCB III, LVSI, and prescription of adjuvant treatment (Table 3). Interestingly, although not statistically significant, there were trends that suggested an advantage of platinum-based NACT (hazard ratio, 0.05; 95% CI, 0.01–1.009; p=0.06) and worse outcome with TILs <30% (hazard ratio, 3.48; 95% CI, 0.96–12.5; p=0.06), and tumor grade III (hazard ratio, 4.2; 95% CI, 1.4–12; p=0.05) on tumor residual. Among significant factors, bilateral BC and LVSI were excluded from the multivariable analysis because of the few patients with bilateral disease and the rate of unknown data for LVSI. On multivariable analysis, multifocal disease (hazard ratio, 3.77; 95% CI, 1.45–9.61; p=0.005) and RCB III (hazard ratio, 3.04; 95% CI, 1.09–9.9; p=0.04) remained significant independent predictors of relapse. Figure 2 presents EFS for patients not achieving pCR with and without multifocal disease and RCB II *vs*. RCB III.

Discussion

TNBC shows more aggressive features and has a poorer prognosis than other types of BC (1). In this subgroup of patients, the achievement of pCR after NACT represents one of the most important indicators of improved outcomes in terms of EFS and OS, whereas residual disease post-NACT is predictive of early recurrences and mortality (4, 5). Our study confirms the significant improved outcome (EFS and OS) of patients who obtained pCR after a standard NACT (mostly anthracycline, taxane, and/or platinum). Particularly, in our cohort, patients with pCR had a 5-year EFS of 90%, while those without pCR had a 5-year EFS of 70% [90% *vs*. 57% in the literature (9)]. On the other hand, the 5-year OS in patients with pCR was 95% compared to 69% of those without pCR (84% *vs*. 47% in the literature (9)). Furthermore, in line with previous literature (1), most of relapses in both our cohorts occurred within the first 5 years after the diagnosis.

The pCR rate after NACT in our study was 43.9%, which is consistent with data in the TNBC literature that typically range from 37% without platinum agents to 52.1% with the addition of platinum compounds (10). Although not statistically significant, also in our population, pCR rate was higher with platinum-based NACT (51.8%) than without platinum agents (42.1%), and this translated into a positive trend in EFS as well. Moreover, according to previous literature (11–14), younger age (<50 years) at diagnosis and high Ki-67 (≥20%) were associated with significantly increased pCR rate.

Up to 10%–20% of TNBC patients are found to carry deleterious germline BRCA1/2 mutations, and mutation prevalence is even higher in younger patients. Particularly, in a recent analysis of Italian TNBC patients diagnosed ≤60 years without breast and/or ovarian family history, BRCA detection rate was 22.6% (15). Although younger age (<50 years) at diagnosis predicts pCR in our analysis, germline mutational status does not significantly impact on pCR rate or on outcomes

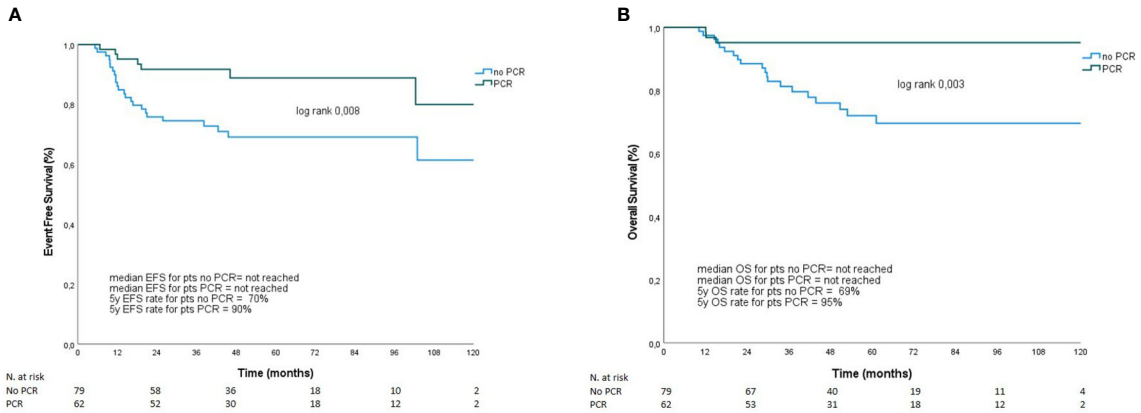


FIGURE 1
EFS **(A)** and OS **(B)** according to pCR. Overall survival and presence or absence of relapse was compared between patients with and without pCR after NACT using the Kaplan–Meier method. Survival estimates were calculated and reported at 5 years, along with their 95% confidence intervals (95% CI), in pCR and no pCR group. **(A)** Patients who obtained pCR after NACT showed 5-year EFS 90% *vs*. 70%, p=0.008. Panel **(B)** represents 5-year OS in patients with and without pCR (95% *vs*. 69%, p=0.003).

10.3389/fonc.2022.1016295

TABLE 3 Univariate and multivariable analyses of EFS in patients not achieving pCR.

	Univariate analysis		*Multivariate analysis*	
Characteristic	***HR (95% CI)***	***P-values***	***HR (95% CI)***	***P-values***
Age at diagnosis				
<50	Ref			
≥50	0.7 (0.31–1.48)	0.34		
Genetic mutation				
No	Ref			
Yes	0.9 (0.6–1.6)	0.96		
BMI				
<25	Ref		Ref	
≥25	0.32 (0.12–0.8)	0.021	0.67 (0.23–1.94)	0.46
Bilateral disease				
No	Ref			
Yes	4.3 (1.3–14.5)	0.01		
Multifocal disease				
No	Ref		Ref	
Yes	3.2 (1.5–6.7)	0.002	3.77 (1.45–9.61)	0.005
MRI				
No	Ref			
Yes	0.7 (0.3–1.4)	0.36		
Histology on tumor biopsy				
Ductal	Ref			
Other	0.8 (0.19–3.4)	0.78		
Grade on tumor biopsy				
II	Ref			
III	1.001 (1–1.001)	0.19		
Ki-67 on tumor biopsy				
<20	Ref			
≥20	0.9 (0.99–1.006)	0.66		
Clinical T stage				
T1–T2	Ref		Ref	
T3–T4	3.4 (1.4–9)	0.02	1.35 (0.49–2.36)	0.07
HER2 low on tumor biopsy				
No	Ref			
Yes	0.7 (0.4–1.1)	0.16		
Clinical N stage				
N0	Ref		Ref	
N+	2.4 (1.1–5)	0.02	3.77 (1.48–9.63)	0.06
Time NACT surgery				
<30 days	Ref			
≥30 days	0.9 (0.94–1)	0.13		
Type surgery				
Conservative	Ref			
Mastectomy	1.8 (0.8–4.5)	0.14		
SLNB				
No	Ref			
Yes	1 (0.97–1.007)	1		
LN assessment				

(Continued)

TABLE 3 Continued

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-values	HR (95% CI)	P-values
≤10	Ref			
>10	1.01 (1.001–1.04)	0.05		
Type of NACT				
Anthra + tax	Ref			
Anthra/Tax	2.98 (0.87–10.1)	0.80		
Plat + other agents	0.41 (0.14–1.13)	0.85		
Anthra/tax	Ref			
Plat + other agents	0.05 (0.01–1.009)	0.06		
Residual cancer burden				
I	Ref			
II	21.8 (0.001–>100)	0.63		
III	0.58 (0.33–089)	0.04		
II	Ref		Ref	
III	8.57 (3.58–21.3)	0.001	3.04 (1.09–9.9)	0.04
TILs on residual tumor				
≥30	Ref			
<30	3.48 (0.96–12.5)	0.06		
Tumor grade on residual tumor				
II	Ref			
III	4.2 (1.4–12)	0.05		
Ki-67 on residual tumor				
<20	Ref			
≥20	0.61 (0.09–3.36)	0.81		
Ki-67 pre- *vs.* post-NACT				
Stable/increased	Ref			
Decreased	1 (0.99–1.01)	0.72		
LVSI on residual tumor				
No	Ref			
Yes	11 (3–36)	0.001		
Adjuvant CHT				
No	Ref		Ref	
Yes	2.9 (1.5–6)	0.03	1.89 (0.21–2.14)	0.54
Time surgery-RT				
≤90 days	Ref			
>90 days	0.99 (0.99–1)	0.09		

BMI, body mass index; MRI, magnetic resonance imaging; T, tumor; HER2, human epidermal growth factor receptor; N, node; NACT, neoadjuvant chemotherapy; SLNB, sentinel lymph node biopsy; LN, lymph node; Anthra, anthracycline-based chemotherapy; Tax, taxane-based chemotherapy; Plat, platinum-based chemotherapy; LVSI, lymphovascular space invasion; CHT, chemotherapy; RT, radiotherapy.

in the non-pCR cohort. According to the local testing criteria in use, 73.2% of patients included in this study underwent genetic testing, and 30% of them presented germline likely pathogenic or pathogenic variants in cancer predisposition genes (24 BRCA1, 4 BRCA2, 1 PALB2, 1 NBN, and 1 ATM). The high detection rate of pathogenic variants in this population is justified by the testing criteria that, before 2016, included only TNBC diagnosed before 40 years of age. On the other hand, the rate of pathogenic variants in the non-BRCA genes could be underestimated because multigene panel testing beyond BRCA genes was introduced in our institution only in 2018. Therefore, the evolution of testing criteria over the years may have

10.3389/fonc.2022.1016295

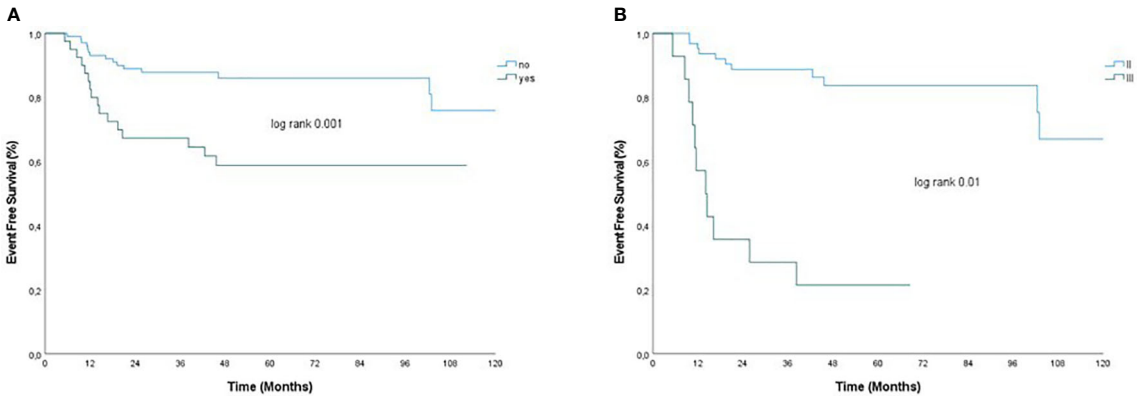


FIGURE 2
EFS by **(A)** multifocal disease and **(B)** RCB in patients without pCR after neoadjuvant chemotherapy. Panel **(A)** presents EFS for patients not achieving pCR with and without multifocal disease (yes/no) and panel **(B)** presents EFS for patients with RCB II *vs.* RCB III.

influenced the detection rate of these gene mutations and may have an impact on the results of our analysis.

The univariable analysis showed a significant association between relapse and BMI > 25, bilateral BC, multifocal disease, clinical T3–T4 stage, clinical N+ stage, RCB III, LVSI, and prescription of adjuvant treatment. Moreover, although not statistically significant likely due to the small sample size, there were trends that also suggested worse outcome with TILs <30% and tumor grade III on tumor residual. With regard to presurgical characteristics, our results are in line with previous literature. Clinical stage at diagnosis was already shown to be one of the major risk factors for recurrence in breast cancer (16–18). Moreover, a recent meta-analysis suggested that overweight is associated with shorter disease-free and overall survival among TNBC patients (19). Among the residual disease features, our findings confirmed the prognostic role of LVSI and TILs in predicting relapse after NACT, as previously showed by an extensive body of literature (20–26). On the other hand, contrary to previous experiences (27–30), we did not observe an increased risk of relapse among patients with high post-treatment Ki-67 value. This discordance may be due to the great variability in Ki-67 evaluation, due to interlaboratory differences in staining methodology, scoring interpretation, and cutoff determination (31). As regards tumor grade, most of the previous studies evaluated tumor grade on biopsies at diagnosis and reported discordant results (32, 33). We did not find a significant association between pretreatment tumor grade and relapse, but a negative trend has been observed in grade III on tumor residual that should be further evaluated in larger cohorts.

Interestingly, in a multivariate model (including BMI > 25, multifocal disease, clinical T3–T4 stage, clinical N+ stage, RCB III, and prescription of adjuvant treatment), BMI, clinical stage, and adjuvant treatment lost significance. The multivariable analysis showed that, in the cohort of patients not achieving pCR, multifocal disease was associated with relapse. For purposes of this analysis, multifocality was defined as the presence of more than one foci in the same breast, regardless of whether they were in the same quadrant or of the distance between the lesions. The role of multifocality in breast cancer is still controversial, and some groups reported a higher rate of relapse and worse outcomes in multifocal tumors, whereas other groups showed that multifocality is not an independent predictor of prognosis in multivariate analysis (33–36).

The multivariable analysis also revealed that RCB III was associated with higher risk of relapse in patients with residual disease after NACT. The RCB score uses the diameter of residual disease, percentage of vital tumor cells, and diameter of the largest involved lymph node to calculate the amount of residual disease. This score has been validated with three distinct prognostic RCB classes in all BC subtypes, with the most significant discriminatory power in TNBC and HER2-positive BC (7, 37, 38). Indeed, our findings are consistent with previous literature and suggest that prospective evaluation of RCB could be considered to become part of standard pathology reporting after NACT, as also recently recommended by the International Collaboration on Cancer Reporting (39). The binary outcome of pCR versus residual disease confers little information, offering no distinction among patients with varied amounts of residual disease. The RCB score has the potential to be used in predicting a patient's residual risk after NACT in a prospective setting, especially given the increasing options for adjuvant therapy in the setting of residual disease.

Age at diagnosis, germline predisposing gene mutations, BMI, breast MRI, histological subtype, grading, Ki-67, HER2 expression, clinical T or N stage, type of breast or axillary

surgery, type of NACT, TILs in the residual tumor, time from NACT end and surgery, and time from surgery to radiation therapy and adjuvant therapy were not independent predictors of relapse in our cohort. Particularly, post-NACT adjuvant systemic treatment did not significantly impact on relapse in our multivariable analyses. In the last decade, several therapies have been investigated as adjuvant strategies in TNBC patients not achieving pCR, including capecitabine and olaparib (40–42). Additionally, immunotherapy is under evaluation in this setting in several clinical trials. Our findings suggest that, despite the addition of further treatments after NACT, the EFS of patient not achieving pCR remains poor, and no improvement has been obtained with the introduction of further adjuvant therapies. According to our findings, the achievement of pCR should remain as the primary aim in these patients and should be pursued by optimizing NACT, for instance by the addition of platinum agents (10, 43) or pembrolizumab (44).

Our study presents some limitations that should be highlighted. First, as a retrospective study, our analysis was limited by selection bias. The sample size is small and derives from a single institution; therefore, our patient population may not accurately represent the patterns of care at other institutions. Additionally, patients received a range of neoadjuvant therapies, and we did not control for the duration of treatment or the delay of dose in this analysis. However, a previous analysis of the ISPY 2 trial suggested that the prognostic association of both pCR and RCB score is strong, regardless of the type of chemotherapybased treatment (45). Finally, some data for each variable were missing in our medical records.

To conclude, our study confirmed the poor prognosis of TNBC patients who do not experience pCR after NACT. The challenge nowadays is to define the treatment paradigm for most of the patients who do not obtain pCR. Waiting for more accurate molecular characterization, multifocality, and RCB remain the most significant risk factors independently predicting relapse among patients without pCR. These data can be used to stratify patients in our clinical practice, potentially guiding treatment decisions and intensifying neo-/adjuvant treatments in patients at higher risk of relapse. Prospective clinical trials are needed to explore novel therapeutic approaches aimed at increasing the rate of pCR and improving adjuvant strategies for this high-risk cohort of patients.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Dataset is available upon reasonable request. Requests to access these datasets should be directed to angela.toss@unimore.it.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Committee of Area Vasta Emilia Nord. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MV, CP, EC, FD, EB, CO, MB, LM, FCa and FCo provided clinical data. MV and AT drafted the manuscript. MC performed the statistical analysis. MV, AT, LC and GT conceived the study and interpreted the results. MV, AT, LC, FP and MD revised the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors especially thank the “Angela Serra Association for Cancer Research” for the support in this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer EP declared a past co-editorship with one of the authors AT to the handling Editor.

Publisher’s note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1016295/full#supplementary-material

References

1. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clin Cancer Res* (2007) 13:4429–34. doi: 10.1158/1078-0432.CCR-06-3045

2. Kojima Y, Tsunoda H. Mammography and ultrasound features of triple-negative breast cancer. *Breast Cancer* (2011) 18:146–51. doi: 10.1007/s12282-010-0223-8

3. Lebert JM, Lester R, Powell E, Seal M, McCarthy J. Advances in the systemic treatment of triple-negative breast cancer. *Curr Oncol* (2018) 25(Suppl 1):S142–50. doi: 10.3747/co.25.3954

4. Liedtke C, Mazouni C, Hess KR, André F, Tordai A, Mejia JA, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *J Clin Oncol* (2008) 26:1275–81. doi: 10.1200/JCO.2007.14.4147

5. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and longterm clinical benefit in breast cancer: The CTNeoBC pooled analysis. *Lancet* (2014) 384:164–72. doi: 10.1016/S0140-6736(13)62422-8

6. von Minckwitz G, Untch M, Blohmer JU, Costa SD, Eidtmann H, Fasching PA, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. *J Clin Oncol* (2012) 30:1796–804. doi: 10.1200/JCO.2011.38.8595

7. Symmans WF, Peintinger F, Hatzis C, Rajan R, Kuerer H, Valero V, et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. *J Clin Oncol* (2007) 25:4414–22. doi: 10.1200/JCO.2007.10.6823

8. Salgado R, Loi S. Tumour infiltrating lymphocytes in breast cancer: Increasing clinical relevance. *Lancet Oncol* (2018) 19:3–5. doi: 10.1016/S1470-2045(17)30905-1

9. Spring LM, Fell G, Arfe A, Sharma C, Greenup R, Reynolds KL, et al. Pathologic complete response after neoadjuvant chemotherapy and impact on breast cancer recurrence and survival: A comprehensive meta-analysis. *Clin Cancer Res* (2020) 26:2838–48. doi: 10.1158/1078-0432.CCR-19-3492

10. Poggio F, Bruzzone M, Ceppi M, Pondé NF, La Valle G, Del Mastro L, et al. Platinum-based neoadjuvant chemotherapy in triple-negative breast cancer: A systematic review and meta-analysis. *Ann Oncol* (2018) 29:1497–508. doi: 10.1093/annonc/mdy127

11. Omarini C, Guaitoli G, Pipitone S, Moscetti L, Cortesi L, Cascinu S, et al. Neoadjuvant treatments in triple-negative breast cancer patients: Where we are now and where we are going. *Cancer Manag Res* (2018) 10:91–103. doi: 10.2147/CMAR.S146658

12. Houssami N, Macaskill P, von Minckwitz G, Marinovich ML, Mamounas E. Meta-analysis of the association of breast cancer subtype and pathologic complete response to neoadjuvant chemotherapy. *Eur J Cancer*. (2012) 48:3342–54. doi: 10.1016/j.ejca.2012.05.023

13. Bagegni NA, Tao Y, Ademuyiwa FO. Clinical outcomes with neoadjuvant versus adjuvant chemotherapy for triple negative breast cancer: A report from the national cancer database. *PloS One* (2019) 14:e0222358. doi: 10.1371/journal.pone.0222358

14. Keam B, Im SA, Lee KH, Han SW, Oh DY, Kim JH, et al. Ki-67 can be used for further classification of triple negative breast cancer into two subtypes with different response and prognosis. *Breast Cancer Res* (2011) 13:R22. doi: 10.1186/bcr2834

15. Toss A, Molinaro E, Venturelli M, Domati F, Marcheselli L, Piana S, et al. BRCA detection rate in an Italian cohort of luminal early-onset and triple-negative breast cancer patients without family history: When biology overcomes genealogy. *Cancers (Basel).* (2020) 12:1252. doi: 10.3390/cancers12051252

16. Keilty D, Nezafat Namini S, Swain M, Maganti M, Cil TD, McCready DR, et al. Patterns of recurrence and predictors of survival in breast cancer patients treated with neoadjuvant chemotherapy, surgery, and radiation. *Int J Radiat Oncol Biol Phys* (2020) 108(3):676–85. doi: 10.1016/j.ijrobp.2020.04.044

17. Ovcaricek T, Frkovic S, Matos E, Mozina B, Borstnar S. Triple negative breast cancer - prognostic factors and survival. *Radiol Oncol* (2010) 45(1):46–52. doi: 10.2478/v10019-010-0054-4

18. Li X, Yang J, Peng L, Sahin. AA, Huo L, Ward KC, et al. Triple-negative breast cancer has worse overall survival and cause-specific survival than non-triple-negative breast cancer. *Breast Cancer Res Treat* (2017) 161(2):279–87. doi: 10.1007/s10549-016-4059-6

19. Harborg S, Zachariae R, Olsen J, Johannsen M, Cronin-Fenton D, Bøggild H, et al. Overweight and prognosis in triple-negative breast cancer patients: a systematic review and meta-analysis. *NPJ Breast Cancer* (2021) 7(1):119. doi: 10.1038/s41523-021-00325-6

20. Denkert C, Von Minckwitz G, Sinn BV, Gade S, Kronenwett R, Pfitzner BM, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol* (2015) 33:983–91. doi: 10.1200/JCO.2014.58.1967

21. Denkert C, Von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: A pooled analysis of 3771 patients treated with neoadjuvant therapy. *Lancet Oncol* (2018) 19:40–50. doi: 10.1016/S1470-2045(17)30904-X

22. Dieci MV, Criscitiello C, Goubar A, Viale G, Conte P, Guarneri V, et al. Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: A retrospective multicenter study. *Ann Oncol* (2014) 25:611–8. doi: 10.1093/annonc/mdt556

23. Wang K, Xu J, Zhang T, Xue D. Tumor-infiltrating lym- phocytes in breast cancer predict the response to chemotherapy and survival outcome: A meta-analysis. *Oncotarget* (2016) 7(28):44288. doi: 10.18632/oncotarget.9988

24. Ochi T, Bianchini G, Ando M, Nozaki F, Kobayashi D, Criscitiello C, et al. Predictive and prognostic value of stromal tumour-infiltrating lymphocytes before and after neoadjuvant therapy in triple negative and HER2-positive breast cancer. *Eur J Cancer* (2019) 118:41–8. doi: 10.1016/j.ejca.2019.05.014

25. Tamura N, Hasebe T, Okada N, Houjoh T, Akashi-Tanaka S, Shimizu C, et al. Tumor histology in lymph vessels and lymph nodes for the accurate prediction of outcome among breast cancer patients treated with neoadjuvant chemotherapy. *Cancer Sci* (2009) 100(10):1823–33. doi: 10.1111/j.1349-7006.2009.01264.x

26. Liu YL, Saraf A, Lee SM, Zhong X, Hibshoosh H, Kalinsky K, et al. Lymphovascular invasion is an independent predictor of survival in breast cancer after neoadjuvant chemotherapy. *Breast Cancer Res Treat* (2016) 157(3):555–64. doi: 10.1007/s10549-016-3837-5

27. Jones RL, Salter J, A'Hern R, Nerurkar A, Parton M, Reis-Filho JS, et al. The prognostic significance of Ki67 before and after neoadjuvant chemotherapy in breast cancer. *Breast Cancer Res Treat* (2009) 116(1):53–68. doi: 10.1007/s10549-008-0081-7

28. von Minckwitz G, Schmitt WD, Loibl S, Müller BM, Blohmer JU, Sinn BV, et al. Ki67 measured after neoadjuvant chemotherapy for primary breast cancer. *Clin Cancer Res* (2013) 19(16):4521–31. doi: 10.1158/1078-0432.CCR-12-3628

29. Tanei T, Shimomura A, Shimazu K, Nakayama T, Kim SJ, Iwamoto T, et al. Prognostic significance of Ki67 index after neoadjuvant chemotherapy in breast cancer. *Eur J Surg Oncol* (2011) 37(2):155–61. doi: 10.1016/j.ejso.2010.10.009

30. Polley MY, Leung SC, McShane LM, Gao D, Hugh JC, Mastropasqua MG, et al. An international Ki67 reproducibility study. *J Natl Cancer Inst* (2013) 105(24):1897–906. doi: 10.1093/jnci/djt306

31. Ataseven B, Lederer B, Blohmer JU, Denkert C, Gerber B, Heil J, et al. Impact of multifocal or multicentric disease on surgery and locoregional, distant and overall survival of 6,134 breast cancer patients treated with neoadjuvant chemotherapy. *Ann Surg Oncol* (2015) 22:1118–27. doi: 10.1245/s10434-014-4122-7

32. Werutsky G, Untch M, Hanusch C, Fasching PA, Blohmer JU, Seiler S, et al. Locoregional recurrence risk after neoadjuvant chemotherapy: A pooled analysis of nine prospective neoadjuvant breast cancer trials. *Eur J Cancer* (2020) 130:92–101. doi: 10.1016/j.ejca.2020.02.015

33. Kennedy WR, Tricarico C, Gabani P, Weiner AA, Altman MB, Ochoa LL, et al. Predictors of distant metastases in triple-negative breast cancer without pathologic complete response after neoadjuvant chemotherapy. *J Natl Compr Canc Netw* (2020) 18:288–96. doi: 10.6004/jnccn.2019.7366

34. Neri A, Marrelli D, Megha T, Bettarini F, Tacchini D, De Franco L, et al. Clinical significance of multifocal and multicentric breast cancers and choice of surgical treatment: A retrospective study on a series of 1158 cases. *BMC Surg* (2015) 15:1. doi: 10.1186/1471-2482-15-1

35. Winters ZE, Horsnell J, Elvers KT, Maxwell AJ, Jones LJ, Shaaban AM, et al. Systematic review of the impact of breast-conserving surgery on cancer outcomes of multiple ipsilateral breast cancers. *BJS Open* (2018) 2:162–74. doi: 10.1002/bjs5.53

36. Symmans WF, Wei C, Gould R, Yu X, Zhang Y, Liu M, et al. Long-term prognostic risk after neoadjuvant chemotherapy associated with residual cancer burden and breast cancer subtype. *J Clin Oncol* (2017) 35:1049–60. doi: 10.1200/JCO.2015.63.1010

37. Yau C, Osdoit M, van der Noordaa M, Shad S, Wei J, de Croze D, et al. Residual cancer burden after neoadjuvant chemotherapy and long-term survival outcomes in breast cancer: A multicentre pooled analysis of 5161 patients. *Lancet Oncol* (2022) 23:149–60. doi: 10.1016/S1470-2045(21)00589-1

38. Bossuyt V, Provenzano E, Symmans WF, Allison KH, Dang C, Gobbi H, et al. *Invasive carcinoma of the breast in the setting of neoadjuvant therapy histopathology reporting guide. 1st edition*. Sydney, Australia: International Collaboration on Cancer Reporting (2022).

39. Parsons HA, Burstein HJ. Adjuvant capecitabine in triple-negative breast cancer: New strategies for tailoring treatment recommendations. *JAMA* (2021) 325:36–8. doi: 10.1001/jama.2020.23371

40. Tutt ANJ, Garber JE, Kaufman B, Viale G, Fumagalli D, Rastogi P, et al. Adjuvant olaparib for patients with BRCA1- or BRCA2-mutated breast cancer. *N Engl J Med* (2021) 384:2394–405. doi: 10.1056/NEJMoa2105215

41. Masuda N, Lee SJ, Ohtani S, Im YH, Lee ES, Yokota I, et al. Adjuvant capecitabine for breast cancer after preoperative chemotherapy. *N Engl J Med* (2017) 376(22):2147–59. doi: 10.1056/NEJMoa1612645

42. Loibl S, O'Shaughnessy J, Untch M, Sikov WM, Rugo HS, McKee MD, et al. Addition of the PARP inhibitor veliparib plus carboplatin or carboplatin alone to standard neoadjuvant chemotherapy in triple-negative breast cancer (BrighTNess): A randomised, phase 3 trial. *Lancet Oncol* (2018) 19:497–509. doi: 10.1016/S1470-2045(18)30111-6

43. Schmid P, Cortes J, Pusztai L, McArthur H, Kümmel S, Bergh J, et al. Pembrolizumab for early triple-negative breast cancer. *N Engl J Med* (2020) 382:810–21. doi: 10.1056/NEJMoa1910549

44. Symmans WF, Yau C, Chen YY, Balassanian R, Klein ME, Pusztai L, et al. Assessment of residual cancer burden and event-free survival in neoadjuvant treatment for high-risk breast cancer: An analysis of data from the I-SPY2 randomized clinical trial. *JAMA Oncol* (2021) 7:1654–63. doi: 10.1001/jamaoncol.2021.3690

45. I-SPY2 Trial Consortium, Yee D, DeMichele AM, Yau C, Isaacs C, Symmans WF, et al. Association of event-free and distant recurrence-free survival with individual-level pathologic complete response in neoadjuvant treatment of stages 2 and 3 breast cancer: Three-year follow-up analysis for the I-SPY2 adaptively randomized clinical trial. *JAMA Oncol* (2020) 6:1355–62. doi: 10.1001/jamaoncol.2020.2535

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 07 December 2022
DOI 10.3389/fonc.2022.990398

OPEN ACCESS

EDITED BY
Svasti Haricharan,
Sanford Burnham Prebys Medical Discovery Institute, United States

REVIEWED BY
Ernestina Marianna De Francesco,
University of Catania, Italy
Cemal Erdem,
Clemson University, United States

*CORRESPONDENCE
Teresa L. Wood
terri.wood@rutgers.edu

†PRESENT ADDRESSES
Alison E. Obr,
Palatin Technologies, Inc, Monmouth Junction, NJ, United States
Yun-Juan Chang,
Office of Cyber Infrastructure &Computational Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, United States

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 09 July 2022
ACCEPTED 10 November 2022
PUBLISHED 07 December 2022

CITATION
Obr AE, Bulatowicz JJ, Chang Y-J, Ciliento V, Lemenze A, Maingrette K, Shang Q, Gallagher EJ, LeRoith D and Wood TL (2022) Breast tumor IGF1R regulates cell adhesion and metastasis: alignment of mouse single cell and human breast cancer transcriptomics. *Front. Oncol.* 12:990398. doi: 10.3389/fonc.2022.990398

Breast tumor IGF1R regulates cell adhesion and metastasis: alignment of mouse single cell and human breast cancer transcriptomics

Alison E. Obr[1†], Joseph J. Bulatowicz[1], Yun-Juan Chang[2†], Virginia Ciliento[1], Alexander Lemenze[3], Krystopher Maingrette[1], Quan Shang[1], Emily J. Gallagher[4], Derek LeRoith[4] and Teresa L. Wood[1*]

[1]Department of Pharmacology, Physiology & Neuroscience, New Jersey Medical School, Rutgers University, Newark, NJ, United States, [2]Office of Advance Research Computing, Rutgers University, Piscataway, NJ, United States, [3]Department of Pathology, New Jersey Medical School, Rutgers University, Newark, NJ, United States, [4]Division of Endocrinology, Diabetes and Bone Diseases, The Samuel Bronfman Department of Medicine, Icahn Sinai School of Medicine at Mt. Sinai, New York, NY, United States

Introduction: The acquisition of a metastatic phenotype is the critical event that determines patient survival from breast cancer. Several receptor tyrosine kinases have functions both in promoting and inhibiting metastasis in breast tumors. Although the insulin-like growth factor 1 receptor (IGF1R) has been considered a target for inhibition in breast cancer, low levels of IGF1R expression are associated with worse overall patient survival.

Methods: To determine how reduced IGF1R impacts tumor phenotype in human breast cancers, we used weighted gene co-expression network analysis (WGCNA) of Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) patient data to identify gene modules associated with low IGF1R expression. We then compared these modules to single cell gene expression analyses and phenotypes of mouse mammary tumors with reduced IGF1R signaling or expression in a tumor model of triple negative breast cancer.

Results: WGCNA from METABRIC data revealed gene modules specific to cell cycle, adhesion, and immune cell signaling that were inversely correlated with IGF1R expression in human breast cancers. Integration of human patient data with single cell sequencing data from mouse tumors revealed similar pathways necessary for promoting metastasis in basal-like mammary tumors with reduced signaling or expression of IGF1R. Functional analyses revealed the basis for the enhanced metastatic phenotype including alterations in E- and P-cadherins.

Discussion: Human breast and mouse mammary tumors with reduced IGF1R are associated with upregulation of several pathways necessary for promoting metastasis supporting the conclusion that IGF1R normally helps maintain a metastasis suppressive tumor microenvironment. We further found that reduced IGF1R signaling in tumor epithelial cells dysregulates cadherin expression resulting in reduced cell adhesion.

KEYWORDS

insulin-like growth factor receptor, metastasis, breast cancer, adhesion, cadherin

Introduction

Metastatic breast cancer is the leading cause of death from breast cancer (1, 2). Several individual genes and associated cellular pathways contribute to a metastatic phenotype but the mechanisms that lead to metastasis are still poorly understood. Receptor tyrosine kinases (RTKs) have been implicated in promoting metastatic properties in tumor cells. RTK domain mutations are not a prominent feature in most cancers; instead, RTK expression level is the general driver of tumorigenesis and metastasis (3–6). A well-known RTK, HER2, has a prominent role in a subclass of breast cancers and has been the focus for successful cancer therapeutics. However, targeting several other RTKs including the epidermal growth factor receptor (EGFR) and the insulin-like growth factor receptor (IGF1R) in breast tumors has been mostly unsuccessful (4, 7, 8). The emerging theme for these receptors is their context- and/or cell-type-dependent functions that change whether they are growth-promoting or growth-inhibiting in the primary tumor or metastatic environment. For example, EGFR signaling promotes growth of primary mammary tumors but suppresses growth of lung metastatic tumors [for review, see (4)]. In the case of the IGF1R, results from mouse models also support a dual function in primary tumor formation and metastasis suppression which may be due to differential actions on proliferation or differentiation depending on the tumor lineage [for review, see (9)].

Expression of IGF1R has been implicated in tumor oncogenesis by promoting tumor cell proliferation and survival (10–12). Due to this oncogenic function, several IGF1R inhibitors have been developed and used in clinical trials. While IGF1R was a clear target, the inhibitors were largely unsuccessful in the clinic (7, 8). There is now evidence that the IGF1R also has tumor or metastasis suppressive functions; IGF1R expression in breast tumors correlates with positive overall patient survival and a more differentiated tumor phenotype (13–15). Consistent with these data, recent analyses using two different patient databases, The Cancer Genome Atlas (TCGA) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC), have revealed low IGF1R expression is associated with undifferentiated, triple-negative breast cancer (TNBC) and worse overall survival (16, 17).

In the present study, we utilized the METABRIC patient database (18) and single-cell RNA sequencing of two IGF1R loss-of-function mouse tumor models to uncover how IGF1R signaling regulates intrinsic epithelial cell signaling to suppress metastasis. We identify key pathways necessary for promoting metastasis including downregulation of immune cell infiltration and function and altered tumor cell phenotype and adherence. Here, we show that IGF1R is required to maintain a metastasis suppressive tumor microenvironment. We further show that reduced IGF1R signaling in tumor epithelial cells dysregulates E- and P-cadherin resulting in reduced cell adhesion.

Materials & methods

Animal models

All animal protocols were approved by the Rutgers University Institutional Animal Care and Use Committee (Newark, NJ) and all experiments were managed in accordance with the NIH guidelines for the care and use of laboratory animals. Animal care was provided by the veterinary staff of the division of animal resources in the New Jersey Medical School Cancer Center of Rutgers Biomedical Health Sciences. The *MMTV-Wnt1* line on an FVB background [FVB.Cg-Tg(Wnt1)1Hev/J] was obtained as a gift from Dr. Yi Li. The *MMTV-Wnt1//MMTV-dnIgf1r* (referred to here as DN-Wnt1) line was described previously (19).

Mice carrying floxed alleles of exon 3 of the *Igf1r* gene (20) were bred with a keratin 8 (K8)-Cre^{ERT} transgenic line (JAX stock #017947) (21) and with the *MMTV-Wnt1* transgenic line to produce female mice that were homozygous for the *Igf1r* floxed alleles and hemizygous for both the K8-Cre^{ERT} and *MMTV-Wnt1* transgenes referred to as K8iKOR-Wnt1 mice.

K8iKOR-Wnt1 tamoxifen dosage paradigm

The tamoxifen dosage paradigm was determined following a developmental study of the effect of tamoxifen on mammary gland development. Three doses of tamoxifen, 5 mg, 2 mg, 1.5 mg or sesame oil were administered once per day for 3 consecutive days in 4-week-old or 8-week-old FVB mice. Four weeks post-injection, mammary gland development was observed using Carnoy's fixative to clear whole mounted mammary glands. Mammary glands from control samples injected with sesame oil demonstrated no significant changes in secondary or tertiary branching compared to naïve glands, while mammary gland development was stunted with the 5 mg dose of tamoxifen administered at 4 weeks of age. Similar to 4 weeks of age, mammary gland branching was stunted at 8 weeks of age with the 5 mg dose of tamoxifen but not with lower tamoxifen doses. Thus, for all tumor studies, tamoxifen (2 mg for 3 consecutive days) was administered at the end of puberty (8 weeks) to avoid disturbing mammary gland development (22, 23) and as confirmed in our studies. Age-matched (8 weeks) females were injected with vehicle sesame oil (control) or tamoxifen for 3 consecutive days to delete the floxed *Igf1r* alleles. Controls for tumor studies included K8-Cre^{ERT} positive females injected with vehicle or K8-Cre^{ERT} negative females injected with tamoxifen. No differences were detected between vehicle and tamoxifen injected controls thus these were combined unless otherwise noted in the methods. Lungs and tumors were harvested when they reached 1.5 cm^3. We confirmed deletion of *Igf1r* K8iKOR-Wnt1 by qRT-PCR for *Igf1r* expression (Supp. Figure 2) and expression of the exon 4 deletion-specific *Igf1r* transcript in tumors and in FAC-sorted luminal epithelial cells.

Tumor latency and growth curves

Wnt1 and K8iKOR-Wnt1 female mice were palpated every five days for tumors beginning at nine weeks of age or 1 wpi sesame oil or tamoxifen. Since no differences in latency were observed between vehicle and tamoxifen injected controls, we combined these animals for these studies. Tumor growth was measured by caliper bi-weekly once a tumor was identified, and the mouse was sacrificed when the tumor reached 1.5 cm^3.

Mammary tumor epithelial cell dissociation

Tumor mammary epithelial cells (MECs) were isolated from Wnt1, DN-Wnt1, and K8iKOR-Wnt1 mice similarly to our prior study (19). Whole tumors were excised and dissociated with the gentleMACs tissue dissociator (130-093-235, protocol m_TDK2) and mouse specific tumor dissociation kit (Miltenyi, 130-096-730). Organoids that retained basement membrane attachments were trypsinized (0.05% Trypsin-EDTA, Gibco) and filtered with a 40 mm cell strainer (BD Biosciences) to isolate a single cell suspension of dissociated tumor MECs. Isolated tumor MECs were counted with a hemocytometer for flow cytometry, FACS, *in vitro* adhesion assays, and cell culture assays.

Sorting of mammary tumor epithelial cells

Tumor MECs from either Wnt1 or DN-Wnt1 mice (n=4) were isolated for single cells as described above with minor adjustments for depletion of unnecessary cells. Red blood cells were lysed with a lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) for 5 minutes. Tumor MECs were resuspended at 10^6 cells/ml in FACS buffer (2% BSA, 2% goat serum in PBS) and immunolabeled with fluorochrome-conjugated cell surface antibodies as described in our previous studies (19). Single cells were prepared for FACS as previously described (24) and sorted at 70 psi using a 70-um nozzle on the Beckton Dickenson FACS Aria directly into PBS.

Flow cytometry analysis of lineage-specific tumor epithelial cells

Tumor MECs from K8iKOR-Wnt1 mice injected with sesame oil or tamoxifen were isolated for single cells as described above. Since no differences in flow cytometry analysis were observed between vehicle and tamoxifen injected controls, we combined these animals. Tumor MECs were immunolabled with fluorochrome-conjugated cell surface antibodies at 1x10^6 cells/100ul FACS buffer as described in our previous studies (17, 19). Cells were labelled for viability using a Live/Dead dye (Invitrogen, L34958) and fixed with 1% paraformaldehyde. Single cells were analyzed using the BD LSRFortessa flow cytometer.

RNA isolation and real-time quantitative PCR

RNA was purified from whole tumor and sorted tumor epithelial cells according to the manufacturer's protocol (Qiagen). RNA concentration and quality was assayed with the NanoDrop ND-1000 (Thermo Scientific). Epithelial cell and sorted tumor epithelial cell cDNA was transcribed according to manufacturer's protocol using SuperScript II (Invitrogen) from total RNA (200 ng). Samples were run in technical triplicate to determine relative gene expression by real-time quantitative PCR (qRT-PCR) detected with SsoAdvanced Universal SYBR Green Supermix (BioRad) using the BioRad CFX96 real-time PCR machine according to manufacturer's

instructions. Transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or Gusb for mouse and ß-actin for human, and data were analyzed using the Q-Gene software (BioTechniques Software Library) (25). Primer oligonucleotide pairs for qRT-PCR are provided (Supp. Table 1).

Histology and immunofluorescence

Tumor tissues and lungs from animals with primary tumors (n=4 per genotype) were drop-fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned at 7 µm. Lung sections from animals with primary tumors were used for hematoxylin and eosin staining. Tumor sections were processed for antigen retrieval for immunofluorescence (IF) as described previously (26). Tissue sections were immunostained with primary antibodies: E-cadherin (1:100; Invitrogen, ECCD-2), P-cadherin (1:100; Invitrogen, MA1-2003), and with species-specific fluorochrome-conjugated secondary antibodies (1:500, Invitrogen).

Fluorescent images were captured using an All-in-One Fluorescent Microscope BZ-X (Keyence, America), and BZ-scientific imaging processing software was used to capture images. At least 5 individual fields were captured at 20X or 40X magnification from tumor sections (n=3 per genotype; 3 sections per genotype averaged). For thicker sections, the Z-stack function was used to capture multiple images on the Z-axis. The Full-focus function was used to select areas at the sharpest focus and obtain the deconvoluted image.

Counting macro and micrometastases in lung sections

Lung tissue from primary and TVI animals were sectioned at 7 µm through the entire lung. For coverage of the entire lung, 3 sections were taken and placed on slides and the next 3 sections were disposed through the entirety of the lung tissue or until reaching 72 individual sections. Representative sections (middle section of each 3 sections) were used for H&E staining. Individual macrometastases were counted by eye and micrometastases were counted at 10X magnification with a brightfield microscope (Olympus Provis AX70) from each H&E-stained slide (n=24).

RNAscope analysis of dominant negative IGF1R expression

RNAScope Multiplex Fluorescent Assay v2 and a human IGF1R probe (Advanced Cell Diagnostics, Inc) was used to determine *dnIGF1R* RNA expression. Tumor tissues were fixed in 4% PFA, paraffin embedded, and sectioned at 7 µm. Tissue samples were deparaffinized and pretreated with hydrogen peroxide, antigen retrieval, and protease plus reagents. (Mild Reagents Timepoint; RNAScope). Tissue sections were incubated at 40°C (Isotemp Incubator, Fisher Scientific) with either Hs-IGF1R-No-XMm probe (Cat No. 471961), Negative probe (Cat No. 320871), or Positive Probe (Cat No 320881). The probe signal was amplified using Amplification Reagents (RNAScope) and signal was developed using the Multiplex FL v2 HRP-C1, HRP blocker, and Opal 620 fluorophore (Akoya Biosciences, FP1495001KT, 1:3000). Sections were incubated with DAPI (RNAScope) and mounted with ProLong Gold Antifade Mounting medium (Invitrogen). Images were captured on the Keyence BZ-X at 40x and 60x magnification.

Tumor epithelial cell *in vitro* adhesion assays

Primary tumors were dissociated as described above and incubated in tissue culture on collagen coated plates for 10 hours. Culture media (DMEM/F12, 5% FBS, insulin (5 µg/mL), EGF (5 ng/mL), hydrocortisone (1 µg/mL), 0.1% gentimicin) was removed and cells in suspension were fixed on slides using a cytospin (Shandon Cytospin 3) for 10 minutes at 1500 rpm for immunofluorescence (IF). Cells attached to the collagen matrix were fixed with 4% PFA for 10 minutes at room temperature for IF analysis or lysed with RLT buffer (Qiagen) for RNA isolation and qRT-PCR analysis as described above.

For IF, cells were processed for staining as previously described (27). Cells were stained with primary antibodies: cytokeratin-8 (1:100; TROMA-I, DSHB) and cytokeratin-14 (1:250; Invitrogen, PA5-16722) and with species-specific fluorochrome-conjugated secondary antibodies (1:500, Invitrogen). To visualize cell nuclei, cells were stained with DAPI (1:10,000 in PBS). Images were captured as described above and cells were manually counted using ImageJ.

Single-cell RNA sequencing

Whole Wnt1 (tamoxifen injected, Cre negative), DN-Wnt1, and K8iKOR-Wnt1 tumors were dissociated as described above and tumor cells were filtered with a 70 µm filter directly after dissociation to collect single cells from the entire tumor. Cells were captured using the 10X Chromium system (10X Genomics) and sequenced with the NextSeq 500 (Illumina). Raw reads were barcode deconvoluted and aligned to the reference genome (mm10) *via* cellranger (v3.1.0). All subsequent processing was performed using the Seurat package within R (v3.1.5). Low quality cells (cells with percentage of reads of mitochondrial

origin >10%, with percentage of reads of ribosomal origin >45%, with <1000 feature counts, with >6000 feature counts) were filtered from the dataset, and read counts were normalized using the scTransform method (28). Samples were integrated with the Seurat integrate function (29) and clustered *via* UMAP according to nearest neighbors. Re-clustering was performed as above on subset clusters based on common annotation types.

WGCNA analysis of METABRIC data for gene module identification

The data generated from 1981 patients within the METABRIC project (18) was used in this investigation. These data were accessed through Synapse (synapse.sagebase.org), including normalized expression data and clinical feature measurements. The associated expression Z scores were downloaded from cBioPortal (30, 31) (https://www.cbioportal.org/). The method of weighted gene co-expression network analysis (WGCNA) (32, 33) was used to identify gene modules with significant statistical association to the phenotypic trait including patient age, tumor size, tumor grade, cancer subtype, and IGF1R expression as Z score.

The analysis was performed within R environment, version 3.6.0, and WGCNA v. 1.68. First, genes with higher expression variance among patient samples (above its quantile) were filtered, resulting in a total of 12394 out of 49576 genes selected. Then, a gene co-expression network was constructed with expression values (normalized) of the selected genes, followed by an adjacency matrix to describe the correlation strength between the nodes. Subsequently, the adjacency matrix was transformed into a topological overlap matrix (TOM), which is a method to quantitatively describe the similarity in nodes by comparing the weighted correlation between two nodes and other nodes. The hierarchical clustering was then applied to identify modules, each containing at least 30 genes (minModuleSize = 30). Finally the eigengene was calculated, the modules were hierarchically clustered, and similar modules were merged (mergeCutHeight = 0.25). A soft-threshold of 6 was chosen which was the lowest power that resulted in a scale free topology fit index to be above 0.9. The correlation between the modules and the clinical data was calculated to identify significant modules correlated with the clinical trait.

Ingenuity pathway analysis

scRNA-seq: Differentially expressed gene sets were identified from the DN-Wnt1 and K8iKOR-Wnt1 compared to Wnt1 mouse tumors for each whole tumor and epithelial cell specific cluster determined from scRNA-seq as described above. These differentially expressed genes were used for IPA enrichment and graphical summary analysis. The top 5 pathways based on significance were plotted by percent genes altered in each pathway. Graphical summaries were generated using the top pathways, cell functions, and target genes identified from differentially expressed genes (DN-Wnt1 vs. Wnt1; K8iKOR-Wnt1 vs. Wnt1) in each cluster.

WGCNA METABRIC analysis: Gene names and expression levels identified from highly correlative co-expression gene modules identified in the WGCNA analysis were uploaded into the IPA software (Qiagen) and analyzed for pathway enrichment. The top 5 pathways based on log-fold change significance for each module were plotted in GraphPad by percentage of total genes up- and down-regulated in each pathway.

Comparison Analysis: Whole tumor gene changes were compared to ME genes where the output is pathway alterations. Here, exact genes were not completely similar, but pathways were comparable.

Statistics

All graphical data were expressed as the mean ± SEM. Statistical comparisons were carried out by GraphPad Prism9 software. The Student's *t*-test or non-parametric Mann-Whitney U test was used for two-group comparisons. Specific comparisons are described in figure legends when necessary. For multiple variable analysis, the One-Way ANOVA with Tukey's Multiple Comparison *post-hoc* test was performed. For the tumor growth curve and *in vitro* adhesion analysis, the non-linear regression least squares regression for slope best fit was used to compare differences between each line. The Chi-Square test was used to determine differences between genotypes in the metastasis table. Power calculations were performed based on pilot data to determine the number of tumor samples necessary using a 2-sided hypothesis test, an a = 0.0025, and 80% power.

Results

Low levels of IGF1R correlate with a metastatic gene signature in breast cancer

Recent analysis of TCGA and METABRIC databases have revealed IGF1R expression is reduced in TNBC (16, 17). Furthermore, low levels of IGF1R predict worse overall patient survival across all breast cancer subtypes (17, 34). Recently, we used the human METABRIC database to stratify low and high IGF1R expressing tumors with lymph node positivity, a readout of early-stage metastasis. These analyses revealed that lymph node positivity is ~20% higher in human breast tumors with low

IGF1R expression versus those with high IGF1R expression (9). Our previous studies reported IGF1R expression levels in human tumors are inversely correlated with several key target genes that alter the tumor microenvironment (17). These expression analyses of human breast tumors with low IGF1R were performed with genes we identified as dysregulated in our mouse tumor model with reduced IGF1R signaling (17, 19). The findings from human and mouse support the hypothesis that low expression of IGF1R could be used to identify gene signatures associated with aggressive breast cancers. Network-based systems biology has become an important method for analyzing high-throughput gene expression data and gene function mining. One of the well-recognized methods, weighted gene co-expression network analysis (WGCNA), generates not only gene co-expression networks, but also a derived partitioning of clusters of genes (modules) and identifies the central players within the modules (32, 33). To independently stratify genes correlated with either low or high IGF1R expression in human breast cancers, we performed a global unbiased WGCNA utilizing the METABRIC database to identify gene expression modules associated with IGF1R expression Z-score, referred to as IGF1R gene set 1 (IGF1R-GS1; Supp. Figure 1A). The modules with the highest correlation were then used to identify relevant pathways using ingenuity pathway analysis (IPA) (IGF1R-GS1; Supp. Figures 1B–H).

Due to the large number of genes and pathways altered in the IGF1R-GS1, we refined our WGCNA analyses to limit the original data set to those genes with the strongest positive or

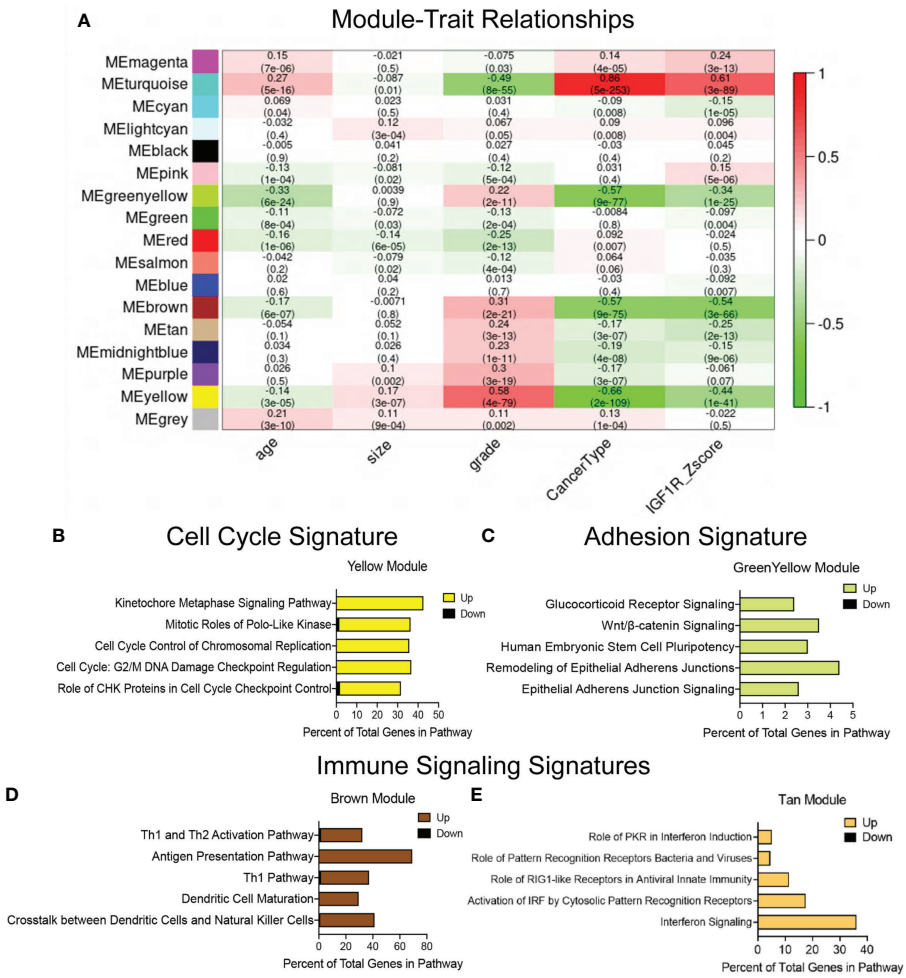
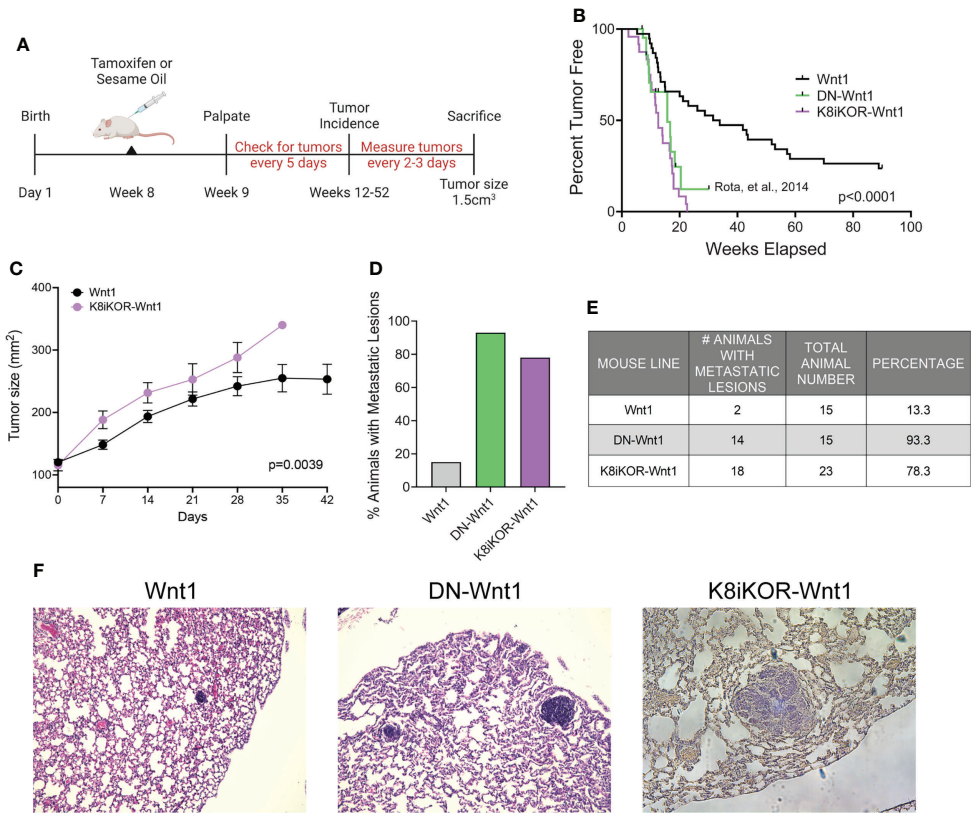


FIGURE 1
Defining gene signatures associated with IGF1R expression and tumor phenotype in human breast cancers. **(A)** Table of refined integrated WGCNA (IGF1R-GS2) showing module and clinical trait association. Each row corresponds to a module eigengene (ME), each column to a clinical measurement. Each cell contains the corresponding correlation and p-value (in parentheses). The table is color-coded by correlation according to the color legend. Green < 0 for negative correlation; Red > 0, for positive correlation. **(B–E)** Top 5 pathways identified by ingenuity pathway analysis (IPA) revealing key signatures in 4 modules inversely correlated with IGF1R expression. (yellow module=cell cycle signature, greenyellow module=adhesion signature, brown and tan modules=immune signaling signatures).

negative correlation to IGF1R expression (Figure 1A). In this refined gene set (IGF1R-GS2), we identified four gene co-expression modules significantly correlated with low IGF1R (correlation score ≤ -0.25), all of which were also associated with high tumor grade and three of which were associated with TNBC. One additional module significantly associated with high IGF1R (correlation 0.61) was also associated with ER+/PR+ breast cancers and low tumor grade (Figure 1A).

We then used IPA on the genes from individual modules identified in IGF1R-GS2 to define the pathways associated with the lowest IGF1R Z-scores. These analyses revealed genes involved in control of cell cycle checkpoint regulation and chromosome replication (yellow, Cell Cycle Signature; Figure 1B), and in epithelial adherens junctions (green-yellow, Adhesion Signature; Figure 1C). The two additional modules associated with low IGF1R contained genes involved in immune cell signaling (brown, tan; Figures 1D, E). Taken together, these findings indicate that reduced IGF1R in breast tumors is associated with alterations in intrinsic tumor epithelial cell pathways as well as extrinsic immune microenvironment signatures that promote metastasis.

A major question that arises from the METABRIC WGCNA is whether there is a causative relationship between IGF1R expression and associated gene alterations and, ultimately, phenotype of breast cancer. We published previously that low IGF1R expression predicts poor patient survival across all breast cancer subtypes (17, 19) suggesting negative functional consequences from loss of IGF1R expression. Our goal in this study was to use mouse models to test the hypothesis from the human data that low IGF1R in breast tumors directly contributes to a metastatic phenotype through dysregulated expression of specific cellular pathways.



MOUSE LINE	# ANIMALS WITH METASTATIC LESIONS	TOTAL ANIMAL NUMBER	PERCENTAGE
Wnt1	2	15	13.3
DN-Wnt1	14	15	93.3
K8iKOR-Wnt1	18	23	78.3

FIGURE 2
Luminal loss of IGF1R decreases tumor latency and increases metastasis. **(A)** Schematic for luminal lineage *Igf1r* knockout. **(B)** Latency curve for tumor development in Wnt1, DN-Wnt1, and K8iKOR-Wnt1 animals. For K8iKOR-Wnt1 animals, tumor latency is weeks post tamoxifen injection. *Statistic:* Mann-Whitney test **(C)** Growth curve after tumors arise until time of euthanization. *Statistic:* Non-linear regression best fit for line slopes. **(D, E)** Graph of the percentage of animals **(D)** and table of number of animals **(E)** with metastatic lesions after establishment of a primary tumor. *Table Statistic*: Chi-square test; p = 0.0251 for Wnt1 vs. DN-Wnt1 and K8iKOR-Wnt1. For Wnt1 controls, vehicle and tamoxifen injected animals were combined as the phenotypes were equivalent. **(F)** Micrograph images showing examples of metastases in H&E stained lung sections from Wnt1, DN-Wnt1 and K8iKOR-Wnt1 mice with primary tumors.

Mammary epithelial cell specific IGF1R deletion promotes Wnt1 driven tumor metastasis

To test how loss of IGF1R alters the primary tumor phenotype, we made use of two distinct mouse models. In one model developed previously in our lab, IGF1R function is reduced through mammary epithelial expression of a dominant-negative human *IGF1R* transgene (MMTV-*dn*IGF1R) in the *MMTV-Wnt1* (Wnt1) basal-like breast cancer tumor model [DN-Wnt1; (19);]. In this mouse line, the loss of IGF1R function results in decreased tumor latency and increased lung metastases, while tumor growth is unchanged (19). To model human breast cancers with low IGF1R expression, we also generated a mammary luminal epithelial lineage-specific *Igf1r* knockout mouse driven from a tamoxifen-inducible Keratin 8 (K8)-Cre, referred to as the K8iKOR line (Figure 2A). Loss of *Igf1r* was verified in mammary epithelial cells (MECs) isolated from hyperplastic glands in 16-week-old virgin K8iKOR-Wnt1 mice compared to control, Wnt1 mice (Supp. Figure 2A). Decreased *Igf1r* gene expression was maintained in tumors of the K8iKOR-Wnt1 line (Supp. Figure 2B).

To determine the effects of luminal epithelial specific *Igf1r* gene deletion in Wnt1-driven mammary tumorigenesis, we assessed tumor latency rates in the K8iKOR-Wnt1 mouse line compared to the control Wnt1 line and to our prior tumor latency data on the DN-Wnt1 mouse line (19). The mean tumor latency of Wnt1 mice was consistent with previous reports (35, 36), where 50% of control Wnt1 animals formed palpable tumors at 41.7 weeks of age (Figure 2B). Tumor latency was significantly decreased in K8iKOR-Wnt1 mice (12.5 weeks after tamoxifen injection, $p<0.0001$) (Figure 2B) similar to the DN-Wnt1 mouse line as previously reported (16.6 weeks, $p<0.0001$) (Figure 2B) (19). Once tumors formed, tumor growth was significantly increased in K8iKOR-Wnt1 compared to control Wnt1 tumors (Figure 2C). These data indicate that decreased expression of *Igf1r* in luminal epithelial cells accelerates tumor initiation as well as tumor growth in the context of elevated Wnt signaling.

Although the Wnt1 tumors model a basal-like TNBC, these tumors have low metastatic potential (35). In contrast, loss of luminal epithelial *Igf1r* in the Wnt1 tumors significantly increased the percentage of animals with lung micrometastases (from 13.3% to 78.3%) similar to the high metastatic rate (93.3%) in the DN-Wnt1 mice (Figures 2D–F). Thus, either reduced *Igf1r* expression or reduced IGF1R function in mammary epithelium promotes metastasis of the primary Wnt1 tumor cells.

Single-cell sequencing of mammary tumors to analyze epithelial IGF1R function in regulating tumor cell heterogeneity

Reduced IGF1R by function or expression results in increased tumor metastasis in the mouse models and aligns with human survival data indicating an inverse relationship between IGF1R expression and overall patient survival (17). The mechanisms by which IGF1R regulates tumor metastasis could include intrinsic epithelial mesenchymal transition (EMT) changes as well as alterations to the tumor microenvironment (TME) secondary to the genetic changes in the tumor epithelium. To reveal underlying mechanisms and cell population changes downstream of alterations in IGF1R, we performed single cell RNA-sequencing (scRNA-seq) on the DN-Wnt1, K8iKOR-Wnt1 and Wnt1 tumors. We initially analyzed scRNA-seq of the whole tumor to profile changes in tumor cell populations when IGF1R is either reduced or attenuated in the tumor epithelium. Wnt1 control, DN-Wnt1 and K8iKOR-Wnt1 tumor cells were plotted together resulting in 17 separate tumor cell populations (Figure 3A). These populations were further defined using cell specific markers resulting in the following distinct cell populations: 7 epithelial, 2 fibroblast (FIBs), 6 macrophage/monocyte (MACs), 1 T-cell, and 1 endothelial (EC) (Figures 3B, C; Supp. Figure 3). Overall, loss of IGF1R expression or function resulted in decreased macrophage and T cell populations and expanded fibroblast populations (Figure 3D). Furthermore, flow cytometry analysis validated increased fibroblasts (Supp. Figures 4A–C) and decreased T cells (17) in tumors with reduced IGF1R function. Ingenuity pathway analysis (IPA) supports the conclusion that loss of IGF1R function promotes an immune evasive TME (Figures 4A, B; Supp. Figure 5). For example, while the cell number is unchanged in MAC Cluster 2 from DN-Wnt1 and K8iKOR-Wnt1 tumors compared to Wnt1 tumors, the immune function pathways are altered with downregulation of genes involved in immune cell activation, antigen presentation, cell adhesion, and infiltration (Figures 4A, B).

Alignment of the immune signature module from the METABRIC data analysis (Figure 1D) revealed several immune signaling pathways in human tumors similarly associated with low IGF1R expression as for the mouse tumors with reduced IGF1R function or expression (Figure 4C). Interestingly, the pathways upregulated in both patient and mouse tumors with reduced IGF1R are important for response to stress signaling and immune cell evasion supporting our prior findings that loss of IGF1R promotes cell stress in human breast cancer cells (17).

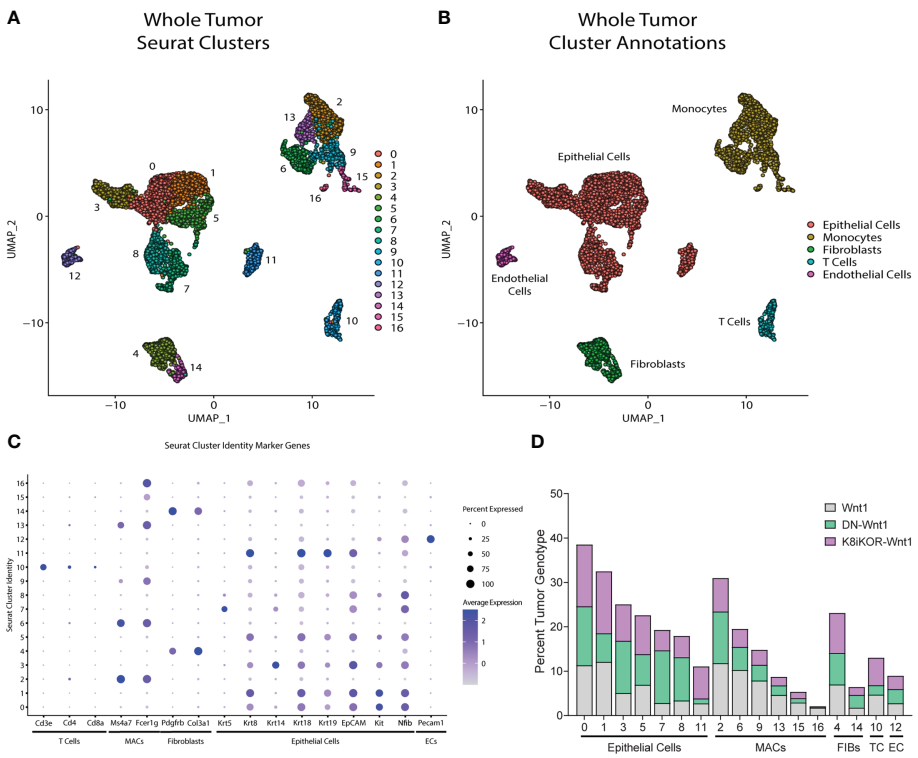


FIGURE 3
Identifying mammary tumor heterogeneity by single cell RNA-sequencing. **(A)** Uniform Manifold Approximation and Projection (UMAP) plot of cells from Wnt1, DN-Wnt1, and K8iKOR-Wnt1 tumors resulting in 17 individual clusters. **(B)** UMAP plot with identification of cluster cell types defined by known markers. **(C)** Dot plot of cell markers. **(D)** Percent tumor genotype graph for each cluster. Clusters are ordered by identified tumor cells. MAC and T-cell populations were generally decreased in DN-Wnt1 and K8iKOR-Wnt1 tumors. (MACs = monocytes/macrophages, TC = T cells, FIBs = fibroblasts, EPI = epithelial cells, EC = endothelial cells).

Expansion of the metastatic tumor epithelial population with reduced IGF1R

We then asked 1) what are the cells from the DN-Wnt1 or K8iKOR-Wnt1 primary tumors that seed lung metastases and 2) what properties of the epithelial cells from the DN-Wnt1 and K8iKOR-Wnt1 tumors promote metastasis? To address these questions, we restricted the scRNA-Seq analysis to the tumor epithelial cell populations. Unsupervised clustering using UMAP resulted in 10 distinct epithelial populations (E0-E9) consisting of 2,543 cells from Wnt1, DN-Wnt1, and K8iKOR-Wnt1 tumors (Figure 5A). Using Seurat and heat map analysis of known epithelial cell population markers (37) we identified the epithelial clusters as: alveolar (E0), luminal (E4,E6,E7,E8), differentiated luminal (E5), luminal progenitor (E1) and basal (E2, E3, E9), one of which (E9) had high expression of the bipotential cell marker Lgr5 (Figures 5B–E; Supp. Figure 6). Importantly, the basal cell clusters (E2,E3), luminal progenitor cluster (E1), and bipotential cluster (E9) were expanded in one or both the K8iKOR-Wnt1 and DN-Wnt1 tumors (Figure 5D). The expansion of the basal and luminal progenitor populations in the IGF1R deficient tumors was supported by flow cytometry analyses of the DN-Wnt1 tumors (19) and the K8iKOR-Wnt1 tumors (Figures 5F–I). Furthermore, the DN-Wnt1 luminal cells were decreased in each cluster suggesting loss of IGF1R function causes luminal cells to either gain basal markers or to de-differentiate into a more basal phenotype. This is supported by data evaluating K14 expression in sorted tumor luminal cells from tumors with reduced IGF1R (Supp. Figure 7). These data revealed an increase in K14 expression in epithelial populations in the IGF1R deficient tumors (Supp. Figure 7A) which was seen only in the sorted luminal epithelial population in the DN-Wnt1 tumors compared to Wnt1 tumors (Supp. Figure 7B).

The bipotential and basal cells are most closely linked to a previously identified metastatic signature (38) (Figure 6A). Expansion of the metastatic bipotential and basal populations is consistent with increased metastasis in the IGF1R deficient tumor models (Figures 2D, E, 6A). Gene Set Enrichment Analysis (GSEA) confirmed enrichment in EMT (Figures 6B, C; Supp. Figure 8) in both the DN-Wnt1 and K8iKOR-Wnt1 tumor epithelial cells, but these analyses began to reveal some distinctions between the two IGF1R deficient tumors. For

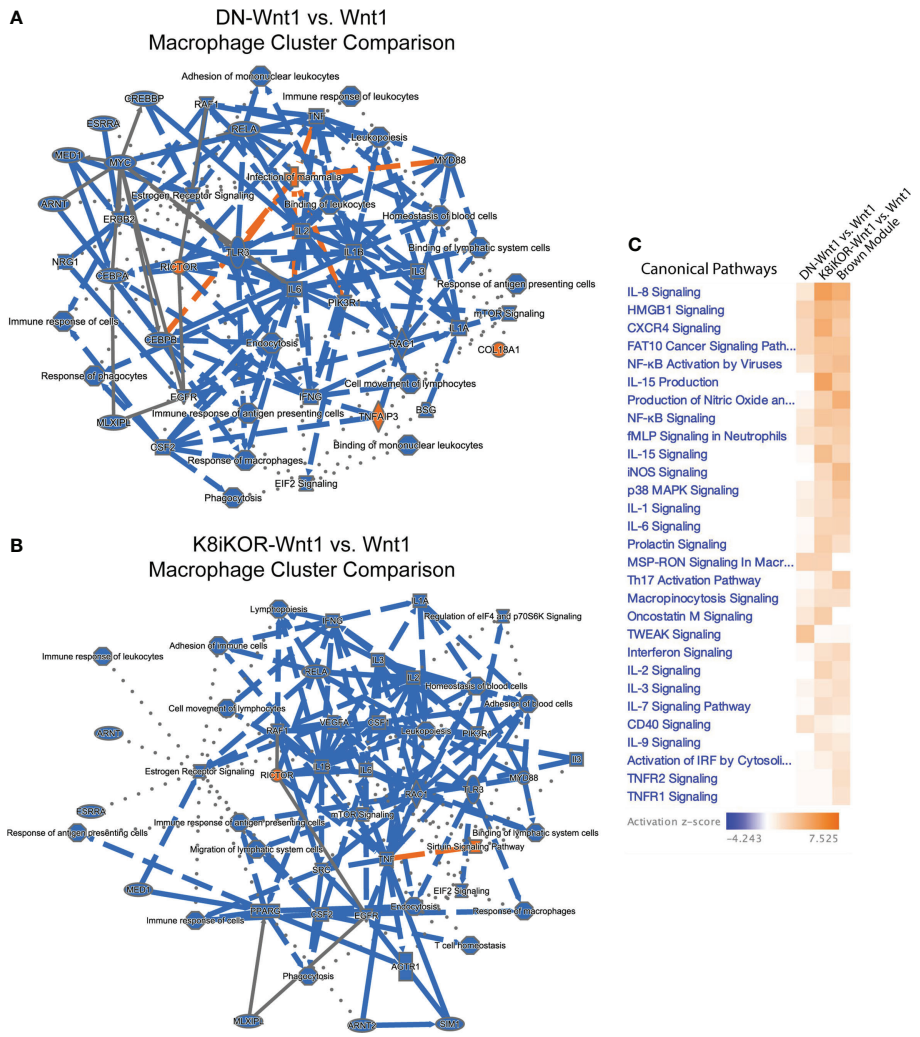


FIGURE 4
(A, B) Macrophage and immune signaling pathways are altered with reduced IGF1R. IPA graphical summary of top pathway alterations in DN-Wnt1 **(A)** or K8iKOR-Wnt1 **(B)** compared to Wnt1 tumors from Cluster 2 (MACs). Blue=downregulated; orange=upregulated. **(C)** IPA canonical pathways heat map of DN-Wnt1 and K8iKOR-Wnt1 compared to Wnt1 tumors and the METABRIC brown (immune signaling signature) module.

example, the basal cluster (E2) and the alveolar/luminal clusters (E0, E7) from the DN-Wnt1 tumors showed increased EMT hallmark signature gene expression in the GSEA analysis, whereas the luminal cluster (E7) and bipotential cluster (E9) in the K8iKOR-Wnt1 tumors had the most pronounced GSEA EMT signatures (Figures 6B, C). This is also consistent with the enrichment dot plot analyses where the strongest EMT profile is seen in the luminal cluster in the DN-Wnt1 tumors and in the bipotential cluster in the K8iKOR-Wnt1 tumors (Supp. Figure 8).

Targeted analysis of the whole tumor using an EMT specific RT2 qPCR assay resulted in increased expression in EMT related genes in DN-Wnt1 tumors compared to Wnt1 tumors (Supp. Figure 9A). IPA further revealed key changes in differentiation, cell migration, invasion, and adherence pathways specific to clusters E0, E2, E7, and E9 in the DN-Wnt1 tumors (Supp. Figures 9B–E). Increased EMT transcripts (Figures 6B, C; Supp. Figures 8, 9) support the conclusion that the epithelial populations are gaining mesenchymal characteristics consistent with increased metastatic potential and increased bipotential populations in the IGF1R deficient tumors.

Cell adherence is altered in tumor epithelial cells with decreased IGF1R function

Recently, the Ewald lab reported E-cadherin loss is required for metastatic invasion, and its re-expression is necessary to

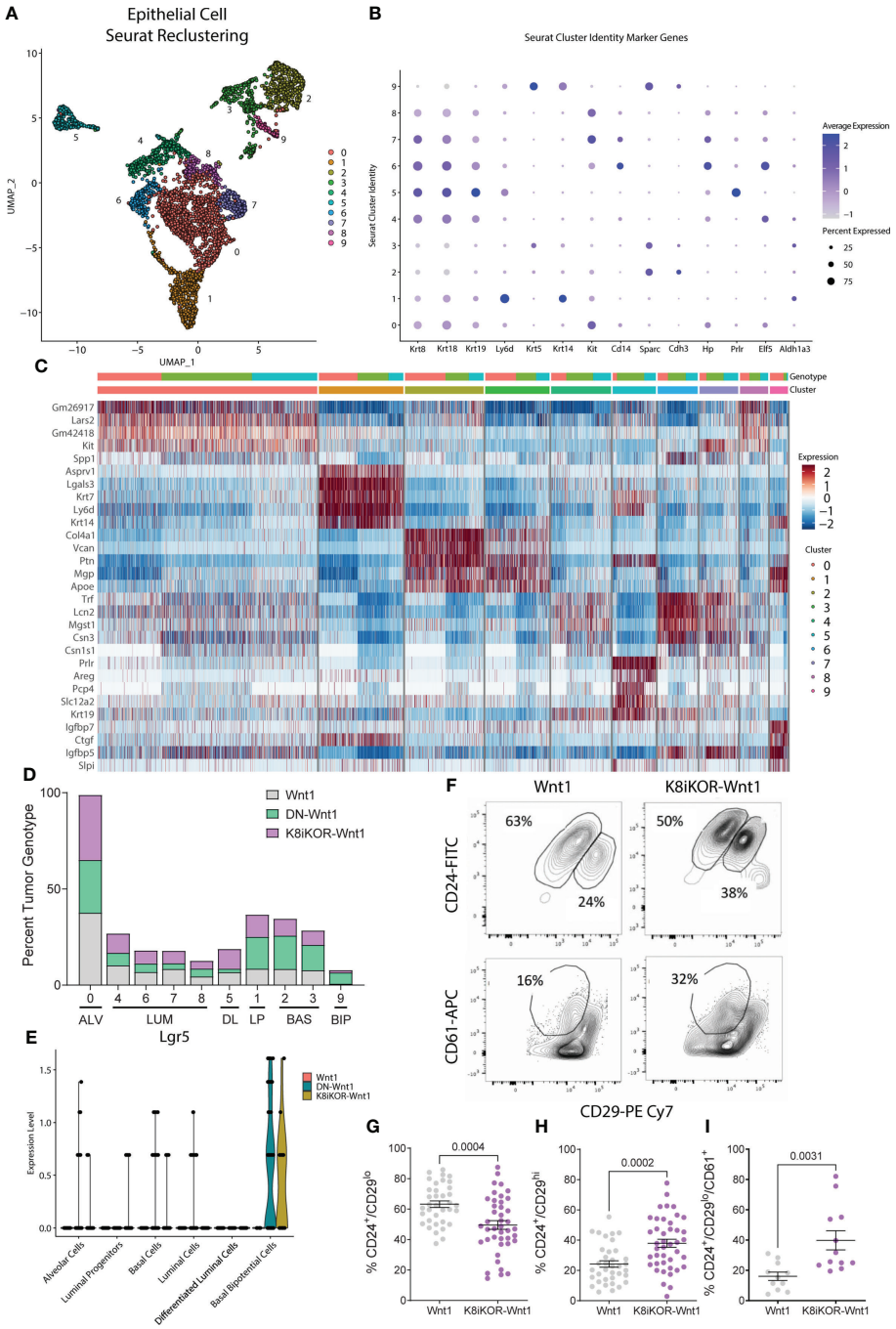


FIGURE 5
Epithelial cell populations are altered with reduced IGF1R. **(A)** UMAP plot of re-clustering of epithelial cells from Wnt1, DN-Wnt1, and K8iKOR-Wnt1 tumors resulting in 13 clusters. **(B)** Dot plot of epithelial cell markers. **(C)** Heat map of top epithelial cell type markers. Top legend: top row=tumor identity: red=Wnt1, green=DN-Wnt1, blue=K8iKOR-Wnt1; Bottom row=epithelial cell cluster. **(D)** Percent tumor genotype graph for each cell cluster labelled with each cell type defined by markers. (ALV=alveolar cell, LUM=luminal cell, DL=differentiated luminal cell, LP=luminal progenitor, BAS=basal cell, BIP=basal bipotential progenitor). **(E)** Violin plot for Lgr5 in each annotated cluster and tumor type. **(F)** Representative contour plots of flow cytometry of the $CD24^{+}/CD29^{lo}$ (luminal) and $CD24^{+}/CD29^{hi}$ (basal) cell populations and $CD24^{+}/CD29^{lo}/CD61^{-}$ (luminal progenitor) cell population in Wnt1 and K8iKOR-Wnt1 tumors. **G-I.** Quantification of luminal **(G)**, basal **(H)**, and luminal progenitor **(I)** populations in Wnt1 and K8iKOR-Wnt1 tumors. Each dot represents an individual tumor. *Statistic:* Unpaired Student's *t*-test.

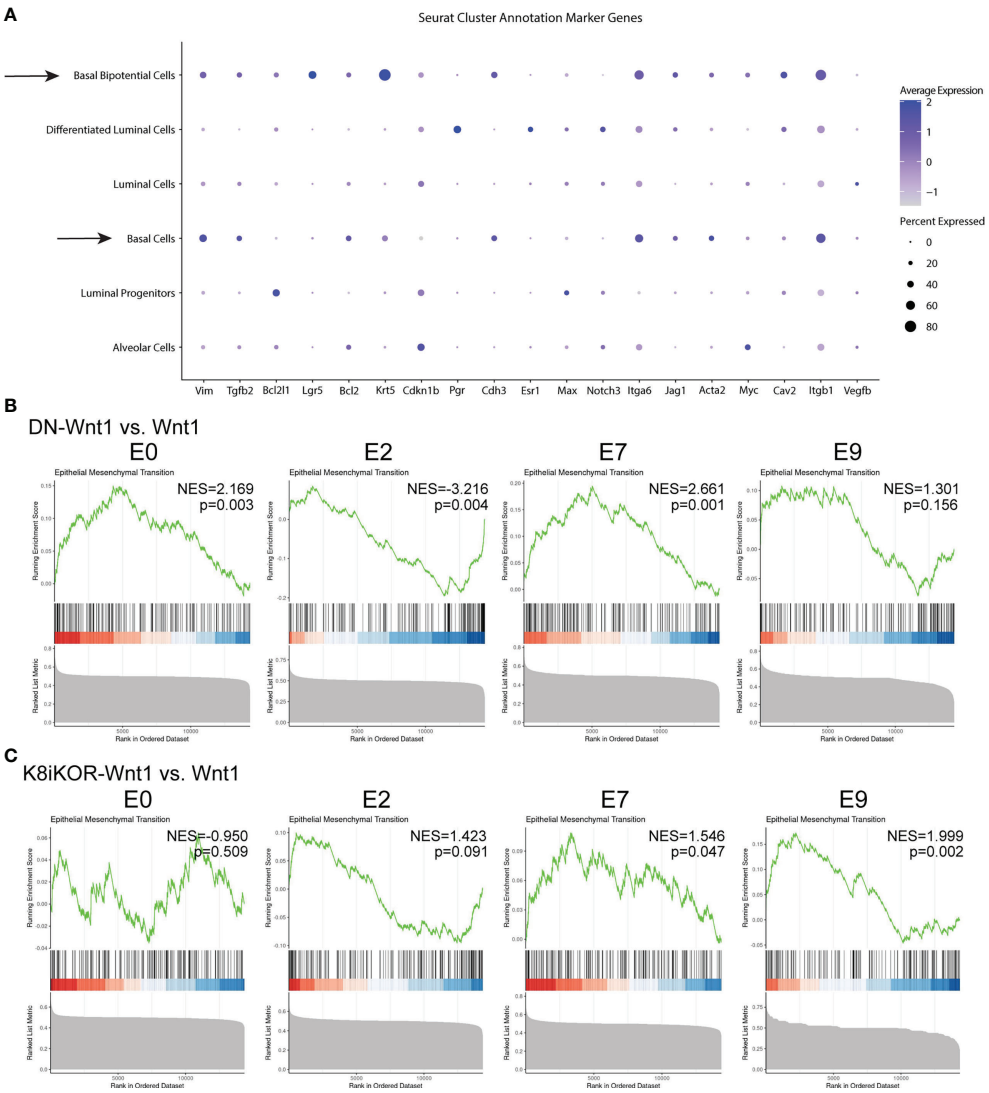


FIGURE 6
A metastatic and EMT phenotype is enhanced in tumors with reduced IGF1R. **(A)** Dot plot from all tumors of alignment with metastatic signature. Arrows depict clusters with high expression of markers indicating metastatic cell type. **(B, C)** GSEA plots for epithelial mesenchymal transition (EMT) hallmark signature in DN-Wnt1 vs. Wnt1 **(B)** and K8iKOR-Wnt1 vs. Wnt1 **(C)** for luminal clusters E0 and E7, basal cluster E2, and bipotential basal cluster E9. NES = normalized enrichment score. P values for each comparison are shown on each plot. Nominal p-value was calculated using 1000 permutations, with FDR correction.

promote metastatic growth (39). To determine whether cadherin expression is altered in tumors with reduced IGF1R, we screened for cadherin expression in each epithelial cluster from the scRNA-Seq data. As expected, luminal cell types had higher E-cadherin (Cdh1) expression whereas basal cell types had higher P-cadherin (Cdh3) and T-cadherin (Cdh13) expression (Figure 7A). Interestingly, bipotential cells have high expression of E-cadherin, as well as P-cadherin (Figures 7A–C) suggesting a less differentiated cell type. Notably, tumor epithelium with reduced IGF1R resulted in increased P-cadherin expression in DN-Wnt1 and K8iKOR-Wnt1 bipotential cells (Figures 7B–C). Furthermore, E-cadherin expression was reduced in both luminal and basal lineages in sorted DN-Wnt1 tumor epithelial cells compared to Wnt1 cells (Figure 7D). To determine if cadherin expression similarly changes with IGF1R expression in patient tumors, we analyzed the METABRIC dataset and identified a positive correlation of E-cadherin with IGF1R expression but an inverse correlation of P-cadherin and IGF1R expression across all breast tumors (Figure 7E).

To test the functional role of adherence gene changes, we measured tumor epithelial cell adherence *in vitro*. Adherence was decreased in DN-Wnt1 and K8iKOR-Wnt1 compared to

Wnt1 primary tumor epithelial cells *in vitro* (Figure 7F). Consistent with these findings, DN-Wnt1 primary tumor epithelial cell clusters and single tumor epithelial cells had decreased adherence to collagen matrix compared to Wnt1 primary tumor cells (Supp. Figures 10A–F). In contrast, there was no significant difference between the K8iKOR-Wnt1 and Wnt1 primary tumor epithelial cells in their ability to adhere to collagen (Supp. Figures 10A–F). Immunofluorescence revealed increased K14^{+} and decreased K8+ cell adherence from DN-Wnt1 compared to Wnt1 primary tumors both in clusters and individual cells (Supp. Figures10F, G). Moreover, the non-adherent cells from the DN-Wnt1 tumors had increased E-cadherin expression indicating it was the luminal epithelial cells with reduced IGF1R signaling that had an adherence deficiency (Supp. Figure 10H). Furthermore, adherent DN-Wnt1 tumor epithelial cells had increased vimentin suggesting mostly basal cell adhesion with reduced IGF1R. These findings support the hypothesis that disruption of IGF1R in both the luminal and basal lineages in the DN-Wnt1 tumors (see below) may be necessary to disrupt adhesion between epithelial cells. These data support changes in adhesion to substrate but without an effect on cell survival. It is also interesting that in our prior study we

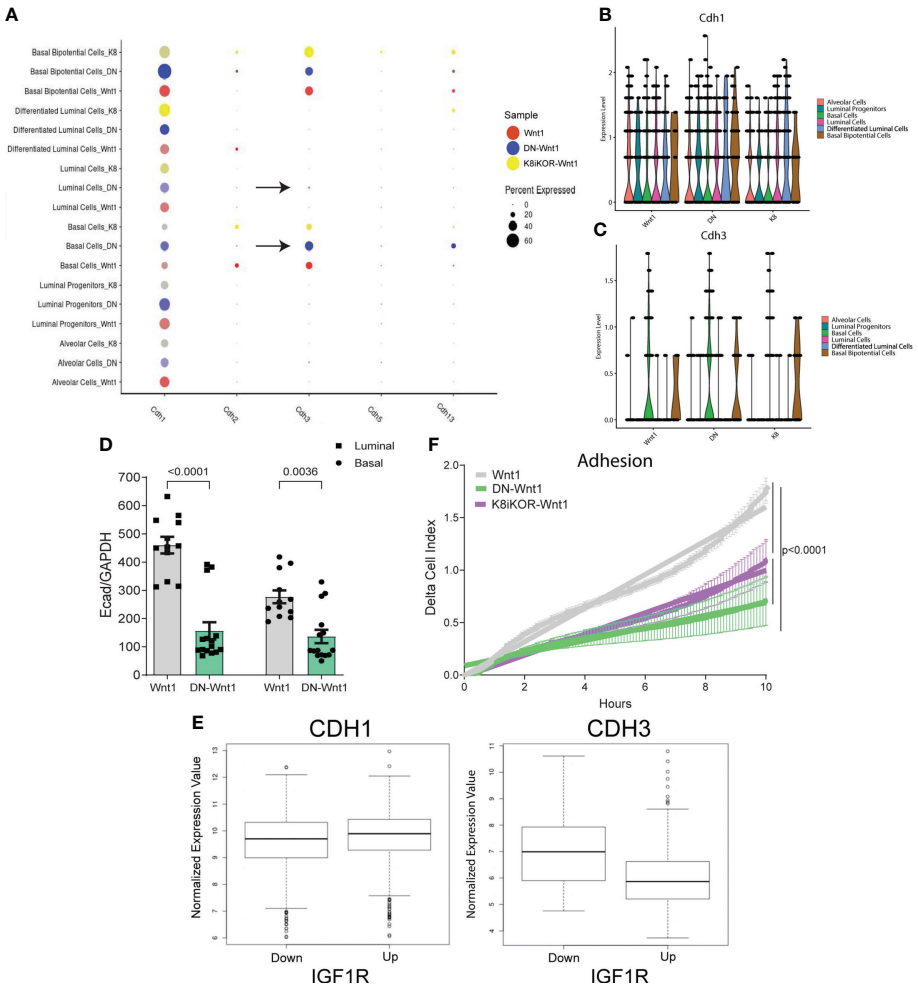


FIGURE 7
Reduced IGF1R function decreases tumor cell adhesion. **(A)** Dot plot of various cadherins expressed in epithelial tumor cell clusters. Arrows depict clusters with an increase in P-cadherin in the DN-Wnt1 tumors. **(B, C)** E-cadherin **(B)** and P-cadherin **(C)** expression in annotated epithelial cell types identified with single-cell sequencing in Wnt1, DN-Wnt1, or K8iKOR-Wnt1 primary tumors. **(D)** RT-PCR for E-cadherin from Wnt1 or DN-Wnt1 sorted luminal and basal epithelial tumor cells. *Statistic:* Non-parametric Mann-Whitney U test. **(E)** METABRIC data analysis for E-cadherin or P-cadherin in patient tumors with low IGF1R (IGF1R z-score < -1) or high IGF1R (IGF1R z-score > 1) ($p < 2.0x10^{-16}$) *Statistic:* Student's *t*-test. **(F)** Measurement of adhesion from Wnt1 (grey), DN-Wnt1 (green), or K8iKOR-Wnt1 (purple) by delta cell index over time for 6 hours using the real-time xCELLigence assay. n=3; *Statistic:* Non-linear regression least squares regression for slope best fit p<0.0001 for Wnt1 control compared to DN-Wnt1 or K8iKOR-Wnt1.

showed that Wnt1 tumor epithelial cells increase tumorsphere formation frequency in non-adherent conditions after IGF1R inhibition (19).

Although the two IGF1R deficient models are similar in having elevated metastases and increased basal and EMT phenotypes in the epithelial cells (Figures 6B, C, Supp. Figures 7,8), they also show some differences particularly in cell adherence phenotypes (Figure 7F, Supp. Figure 10). Two possible explanations for the discrepancy in the adherence phenotype and gene expression pathway alterations between the two models are the mode and lineage specificity of IGF1R disruption. The DN-Wnt1 model expresses a dominant-negative IGF1R transgene that inhibits IGF1R tyrosine kinase function. In this model, the MMTV promoter is active early in the mammary epithelial lineage such that both lineages express the transgene (40). RNAscope immunofluorescence analysis for the human *dnIGF1R* transgene confirmed expression in hyperplastic mammary glands and tumors from the DN-Wnt1 mice (Supp. Figures 11A–H). We further verified the expression of the *dnIGF1R* transgene in both luminal and basal epithelial lineages by performing qRT-PCR for the human *dnIGF1R* transgene in tumor epithelial cells following FACS (Supp. Figure 11I). In contrast to the DN-Wnt1 model, the K8iKOR-Wnt1 model has an *Igf1r* gene deletion specifically in the K8 luminal lineage. Thus, disruption of receptor signaling versus complete loss of the receptor could lead to different phenotypes as well as the disruption of the IGF1R in both epithelial lineages compared to the luminal lineage only.

Cell adherence is dysregulated by enhanced P-cadherin expression in epithelial cells with reduced IGF1R function

Since cadherin gene expression levels are altered with reduced IGF1R, we further analyzed protein levels in tumor tissues to correlate with gene expression. Immunostaining of tumors showed decreased E-cadherin and increased P-cadherin protein expression in DN-Wnt1 and K8iKOR-Wnt1 primary tumors compared to Wnt1 tumors (Figures 8A–J). Interestingly, total E-cadherin expression was altered primarily at the protein level in the DN-Wnt1 tumors. Importantly, co-expression of E-cadherin and P-cadherin was increased in DN-Wnt1 and K8iKOR-Wnt1 tumors (Figures 8G–J). Co-expression of P-cadherin with E-cadherin in the primary tumor is a marker of more aggressive, metastatic breast tumors (41–44). Thus, reduced IGF1R was associated with altered E-cadherin and P-cadherin in tumor epithelial cells.

To test the functional role of altered E-cadherin and P-cadherin in cells with attenuated IGF1R, we first transiently re-expressed E-cadherin in DN-Wnt1 primary tumor epithelial

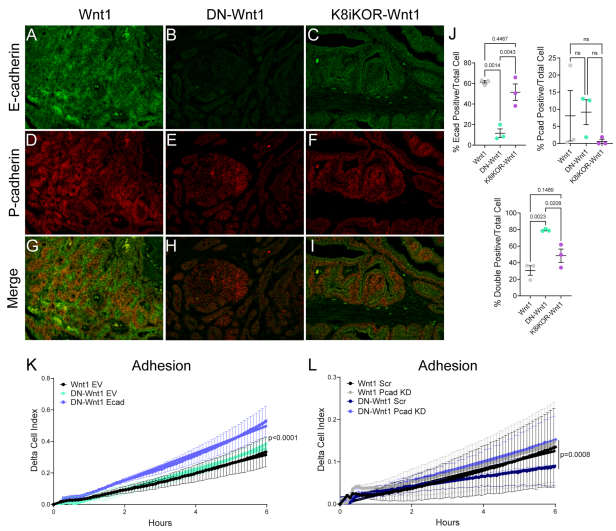


FIGURE 8
Altered cadherin expression in tumors with reduced IGF1R. **(A–I)** Representative images of E-cadherin (green) or P-cadherin (red) immunostaining in Wnt1 **(A, D, G)**, DN-Wnt1 **(B, E, H)**, and K8iKOR-Wnt1 **(C, F, I)** primary tumors. **(J)** E-cadherin, P-cadherin, and double positive cell count graphs of primary tumors. *Statistic:* One-Way ANOVA with Tukey's Multiple Comparison *post-hoc* test. **K.** Adhesion (delta cell index) over time in Wnt1 or DN-Wnt1 primary tumors with empty vector (EV) or E-cadherin overexpression (Ecad). n = 3; *Statistic:* Non-linear regression. **(L)** Adhesion (delta cell index) over time in Wnt1 or DN-Wnt1 with P-cadherin knockdown (Pcad KD). n = 3; *Statistic:* Non-linear regression.

cells and measured cell adhesion *in vitro*. Overexpression of E-cadherin increased epithelial cell adhesion compared to empty vector control (Figure 8K). Furthermore, reducing P-cadherin in DN-Wnt1 primary tumor epithelial cells significantly increased tumor adhesion restoring adhesion back to the level of the Wnt1 tumor cells (Figure 8L). Thus, altering cadherins in DN-Wnt1 primary tumor epithelial cells rescues the compromised adherence suggesting these changes in E- and P-cadherins due to reduced IGF1R are necessary for metastasis.

Discussion

A major question in cancer biology is how do primary tumor cells metastasize to another site? Here we show loss of IGF1R in the primary tumor promotes metastasis by modulating cadherin expression and altering epithelial cell properties to decrease cellular adhesion. While it is well established that epithelial cells gain mesenchymal cell properties to migrate out of the primary tumor (45–49), several recent studies have shown only a subset of mesenchymal properties are necessary for migration and invasion referred to as partial EMT (50–53). While original dogma was that the metastatic process occurs by single tumor epithelial cell migration and invasion, recent observations of collective epithelial cell migration have presented a new mechanism for metastasis that relies on interactions between a mesenchymal-like leader cell with other epithelial cells in the primary tumor (45). Thus, understanding how cell-cell interactions are regulated both in the primary tumor and at distant sites of colonization is critical to determining metastatic potential of tumor cells.

Loss of E-cadherin is a hallmark of EMT and necessary for basal cells to adapt to becoming leader metastatic cells (46). The Ewald lab previously described a process by which the transition of E-cadherin expression is critical for collective invasion (39). Here, we have shown E-cadherin expression is decreased in mouse models with reduced function or expression of IGF1R to drive collective invasion. Prior reports have also linked E-cadherin and IGF1R in breast cancers. Proteomic screening and network analyses of breast cancer cell lines stimulated with either IGF-1 or insulin suggested signaling interactions between the two pathways (54). In subsequent validation of these analyses, the authors demonstrated that knockdown of E-cadherin augmented p-Akt levels particularly in cells stimulated with IGF-1 (54). A subsequent report showed direct interaction between IGF1R and E-cadherin and similarly showed that loss of E-cadherin increased activation of IGF1R signaling (55). Our data seemingly contradict these findings; however, our studies analyzed effects on E-cadherin and adhesion from the perspective of IGF1R reduction rather than the reverse. It is possible that the interaction between the two proteins helps stabilize E-cadherin but also suppresses IGF1R signaling. Our data also reveal that attenuation or reduced IGF1R in the Wnt1-driven tumors augments P-cadherin expression. Interestingly, recent reports have shown acquisition of P-cadherin is necessary for tumor cells to become metastatic. More importantly, the co-expression of P-cadherin and E-cadherin is critical for enhanced metastasis and suggests these cells are exhibiting a partial EMT phenotype. The co-expression of P-cadherin and E-cadherin and a partial EMT phenotype in IGF1R-reduced Wnt1 tumors suggests increased metastatic properties of these tumor cells.

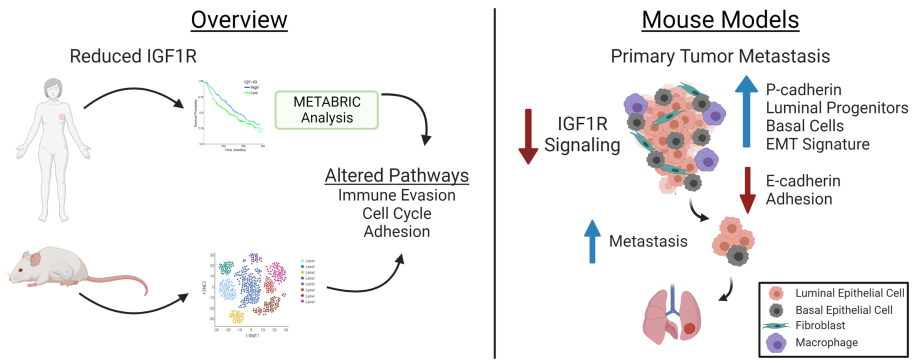


FIGURE 9
Model for how Reduced IGF1R in Human Breast Tumors (left) and in Mouse Basal-Like Mammary Tumors results in Enhanced Metastasis. The overview panel (left) summarizes human breast cancer data showing inverse correlation between IGF1R expression and patient survival and lymph node positivity [see (9, 17)] and from METABRIC data analyses in the current manuscript showing low tumor IGF1R expression is correlated with gene expression indicating increased tumor cell invasion properties and cell cycle and decreased cell adhesion. Similar pathways were identified from scRNA-Seq of the tumors in the mouse models with reduced IGF1R signaling or expression. Analyses of the mouse models (right) revealed decreased IGF1R signaling in primary tumor epithelium resulted in increased lung metastases and alterations associated with metastasis including increased P-cadherin in E-cadherin-positive cells, increased EMT signatures in luminal and basal cell populations and in decreased E-cadherin and cell adhesion. Created with BioRender.com.

While loss of IGF1R is sufficient to drive a partial EMT phenotype and collective invasion to promote metastasis, alterations in the tumor microenvironment may also be required for increased tumor extravasation. Our previous studies showed heightened cell stress driven by attenuated IGF1R resulted in immune cell evasion and a pro-metastatic tumor microenvironment (17). Single-cell RNA sequencing analysis of tumors with reduced IGF1R recapitulate these previous data by showing depletion of immune cell populations and alterations in immune cell function genes and pathways. Furthermore, stroma changes shown in our previous study (17) could be attributed to expansion of fibroblast populations in tumors with reduced IGF1R function (Figure 3D; Supp. Figure 4). Taken together, it is clear that loss of IGF1R in mammary tumors alters the microenvironment to promote metastasis.

One question that arises from inhibiting IGF1R in our tumor models is whether there may be compensatory expression or activation of the insulin receptor (INSR). In our initial publication on development of the DN-Wnt1 tumor model (19), we found that P/T Akt and P/T Erk were reduced in normal mammary epithelial cells expressing the DN-IGF1R. Moreover, P/T IRS-1 was decreased in the DN-Wnt1 tumors compared to Wnt1 tumors. These data argue against compensation by increased INSR signaling. However, we did see a shift in the *Insr-A:Insr-B* isoform ratio as well as increased expression of *Igf2* mRNA in the DN-Wnt1 tumors supporting an IGF-II/INSR-A signaling loop. From the scSeq data in the current analyses, we observed a reduction in *Insr* mRNA expression in luminal clusters 5,7 and 8 in both the DN-Wnt1 and K8iKOR-Wnt1 tumors compared to Wnt1 tumors. This was confirmed by RT-PCR analyses (not shown). However, western blot analyses of total INSR expression indicated no significant change in INSR at the protein level (analyzed in DN-Wnt1 tumors vs Wnt1 tumors; not shown). Interestingly, TCGA analysis of human breast tumors revealed a positive correlation between IGF1R and INSR expression (56). Thus, while we cannot entirely rule out an increase in INSR activation in the IGF1R deficient tumor models, there is not a compensatory increase in expression of the INSR.

While a similar metastatic process is observed in the DN-Wnt1 and K8iKOR-Wnt1 primary tumor models, the scRNA-seq analysis revealed clear differences in the genomic profile of the primary tumor cells in these models. Similarly, minor phenotypic differences have been observed when measuring cell adherence. There are two key differences in these models that likely contribute to these findings: 1) the DN-Wnt1 model attenuates the receptor activity whereas the K8iKOR-Wnt1 model is a gene knockout in the luminal epithelium, and 2) the *dnIGF1R* transgene is expressed in luminal and basal epithelial cells blocking the receptor function in all mammary epithelium, whereas receptor expression is decreased only in the luminal epithelial cells in the K8iKOR-Wnt1 model leaving the basal cell IGF1R intact. Potentially, the loss of IGF1R function in both luminal and basal epithelial cells may lead to the heightened model phenotype because of reduced adherence. These findings emphasize modeling importance.

It is clear from the spontaneous tumor models attenuated or loss of IGF1R decreases tumor latency and increases metastasis. These results are consistent with the clinical data where trials inhibiting IGF1R have been unsuccessful. The interconnectedness of the tumor epithelium and microenvironment is highly complex. The advantage of our models is the ability to study stochastic tumor progression in the context of the microenvironment which reveals this complex tumor biology. Importantly, the mouse modeling data aligns with the human gene expression and pathway analyses (Figure 9) and provides a basis for understanding why loss of IGF1R in human breast cancers is associated with a worse outcome.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found on: https://www.ncbi.nlm.nih.gov/geo/, GSE182236 and on https://osf.io/9mkc4/.

Ethics statement

The animal study was reviewed and approved by Rutgers University IACUC.

Author contributions

AO performed the majority of the experiments and statistical analyses, participated in the study design and wrote the manuscript. JB performed metastases quantification, qRT-PCR for *Igf1r* deletion in sorted cell populations, participated in the *in vitro* adhesion assays and study design and in manuscript and figure revisions. Y-JC performed the WGCNA METABRIC analysis. VC performed the initial analyses on the K8iKOR-Wnt1 mouse tumor line. AL performed the scRNA-Seq analyses. KM performed metastases quantification, RNAScope and participated in the *in vitro* adhesion assays and study design. QS performed mouse genotyping, tamoxifen tests, gland analyses and tumor harvesting. EJG and DL contributed to results interpretation and manuscript editing. TLW is the principal investigator for this project and was involved in study design, data analysis, manuscript editing and submission. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by Public Health Service National Institutes of Health grants NCI R01CA204312 (TW) and NCI R01CA128799 (DL), New Jersey Commission on Cancer Research Postdoctoral Fellowship DFHS15PPC039 and American Cancer Society-Fairfield County Roast Postdoctoral Fellowship 130455-PF-17-244-01-CSM (AO).

Acknowledgments

We thank Dr. Sukhwinder Singh of the NJMS Flow Cytometry and Immunology Core Laboratory for the assistance with flow cytometry analysis and sorting, the Office of Advanced Research Computing (OARC) at Rutgers University under NIH 1S10OD012346-01A1 for the critical work made possible through access to the Perceval Linux cluster, BioRender.com for access to create the graphical abstract, and Dr. Yi Li for providing the *MMTV-Wnt1* mice.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.990398/full#supplementary-material

References

1. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science* (2011) 331(6024):1559–64. doi: 10.1126/science.1203543

2. Wang L, Zhang S, Wang X. The metabolic mechanisms of breast cancer metastasis. *Front Oncol* (2020) 10:602416. doi: 10.3389/fonc.2020.602416

3. Lero MW, Shaw LM. Diversity of insulin and IGF signaling in breast cancer: Implications for therapy. *Mol Cell Endocrinol* (2021) 527:111213. doi: 10.1016/j.mce.2021.111213

4. Ali R, Wendt MK. The paradoxical functions of EGFR during breast cancer progression. *Signal Transduct Target Ther* (2017) 2:16042. doi: 10.1038/sigtrans.2016.42

5. Carvalho I, Milanezi F, Martins A, Reis RM, Schmitt F. Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. *Breast Cancer Res* (2005) 7(5):R788–95. doi: 10.1186/bcr1304

6. Nassar A, Khoor A, Radhakrishnan R, Radhakrishnan A, Cohen C. Correlation of HER2 overexpression with gene amplification and its relation to chromosome 17 aneuploidy: a 5-year experience with invasive ductal and lobular carcinomas. *Int J Clin Exp Pathol* (2014) 7(9):6254–61.

7. Yang Y, Yee D. Targeting insulin and insulin-like growth factor signaling in breast cancer. *J mammary gland Biol neoplasia* (2012) 17(3-4):251–61. doi: 10.1007/s10911-012-9268-y

8. Boone DN, Lee AV. Targeting the insulin-like growth factor receptor: developing biomarkers from gene expression profiling. *Crit Rev oncogenesis* (2012) 17(2):161–73. doi: 10.1615/CritRevOncog.v17.i2.30

9. Bulatowicz JJ, Wood TL. Activation versus inhibition of IGF1R: A dual role in breast tumorigenesis. *Front Endocrinol (Lausanne)* (2022) 13:911079. doi: 10.3389/fendo.2022.911079

10. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* (2008) 8(12):915–28. doi: 10.1038/nrc2536

11. Belfiore A, Frasca F. IGF and insulin receptor signaling in breast cancer. *J mammary gland Biol neoplasia* (2008) 13(4):381–406. doi: 10.1007/s10911-008-9099-z

12. Lann D, LeRoith D. The role of endocrine insulin-like growth factor-I and insulin in breast cancer. *J mammary gland Biol neoplasia* (2008) 13(4):371–9. doi: 10.1007/s10911-008-9100-x

13. Papa V, Gliozzo B, Clark GM, McGuire WL, Moore D, Fujita-Yamaguchi Y, et al. Insulin-like growth factor-I receptors are overexpressed and predict a low risk in human breast cancer. *Cancer Res* (1993) 53(16):3736–40.

14. Bonneterre J, Peyrat JP, Beuscart R, Demaille A. Prognostic significance of insulin-like growth factor 1 receptors in human breast cancer. *Cancer Res* (1990) 50 (21):6931–5.

15. Yerushalmi R, Gelmon KA, Leung S, Gao D, Cheang M, Pollak M, et al. Insulin-like growth factor receptor (IGF-1R) in breast cancer subtypes. *Breast Cancer Res Treat* (2012) 132(1):131–42. doi: 10.1007/s10549-011-1529-8

16. Farabaugh SM, Chan BT, Cui X, Dearth RK, Lee AV. Lack of interaction between ErbB2 and insulin receptor substrate signaling in breast cancer. *Cell Commun Signal* (2016) 14(1):25. doi: 10.1186/s12964-016-0148-8

17. Obr AE, Kumar S, Chang YJ, Bulatowicz JJ, Barnes BJ, Birge RB, et al. Insulin-like growth factor receptor signaling in breast tumor epithelium protects cells from endoplasmic reticulum stress and regulates the tumor microenvironment. *Breast Cancer Res* (2018) 20(1):138. doi: 10.1186/s13058-018-1063-2

18. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* (2012) 486(7403):346–52. doi: 10.1038/nature10983

19. Rota LM, Albanito L, Shin ME, Goyeneche CL, Shushanov S, Gallagher EJ, et al. IGF1R inhibition in mammary epithelia promotes canonical wnt signaling and Wnt1-driven tumors. *Cancer Res* (2014) 74(19):5668–79. doi: 10.1158/0008-5472.CAN-14-0970

20. Cadoret A, Desbois-Mouthon C, Wendum D, Leneuve P, Perret C, Tronche F, et al. C-myc-induced hepatocarcinogenesis in the absence of IGF-I receptor. *Int J Cancer* (2005) 114(4):668–72. doi: 10.1002/ijc.20805

21. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. *Nature* (2011) 479(7372):189–93. doi: 10.1038/nature10573

22. Rios AC, Fu NY, Lindeman GJ, Visvader JE. *In situ* identification of bipotent stem cells in the mammary gland. *Nature* (2014) 506(7488):322–7. doi: 10.1038/nature12948

23. Shehata M, van Amerongen R, Zeeman AL, Giraddi RR, Stingl J. The influence of tamoxifen on normal mouse mammary gland homeostasis. *Breast Cancer Res* (2014) 16(4):411. doi: 10.1186/s13058-014-0411-0

24. Smalley MJ, Kendrick H, Sheridan JM, Regan JL, Prater MD, Lindeman GJ, et al. Isolation of mouse mammary epithelial subpopulations: a comparison of leading methods. *J mammary gland Biol neoplasia* (2012) 17(2):91–7. doi: 10.1007/s10911-012-9257-1

25. Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* (2002) 32(6):1372–4, 6, 8-9.

26. Sun Z, Shushanov S, Leroith D, Wood TL. Decreased IGF type 1 receptor signaling in mammary epithelium during pregnancy leads to reduced proliferation, alveolar differentiation, and expression of insulin receptor substrate (IRS)-1 and IRS-2. *Endocrinology* (2011) 152(8):3233–45. doi: 10.1210/en.2010-1296

27. Obr AE, Grimm SL, Bishop KA, Pike JW, Lydon JP, Edwards DP. Progesterone receptor and Stat5 signaling cross talk through RANKL in mammary epithelial cells. *Mol Endocrinol* (2013) 27(11):1808–24. doi: 10.1210/me.2013-1077

28. Hafemeister C, Satija R. Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol* (2019) 20(1):296. doi: 10.1186/s13059-019-1874-1

29. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM3rd, et al. Comprehensive integration of single-cell data. *Cell* (2019) 177(7):1888–902 e21. doi: 10.1016/j.cell.2019.05.031

30. Cerami, et al. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. *Cancer Discovery* (2012) 401.

31. Gao, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* (2013) 6:pl1.

32. Langfelder P, Horvath S. WGCNA: an r package for weighted correlation network analysis. *BMC Bioinf* (2008) 9:559. doi: 10.1186/1471-2105-9-559

33. Langfelder P, Horvath S. Fast R functions for robust correlations and hierarchical clustering. *J Stat Software* (2012) 46(11):1–17. doi: 10.18637/jss.v046.i11

34. Fu P, Ibusuki M, Yamamoto Y, Hayashi M, Murakami K, Zheng S, et al. Insulin-like growth factor-1 receptor gene expression is associated with survival in breast cancer: a comprehensive analysis of gene copy number, mRNA and protein expression. *Breast Cancer Res Treat* (2011) 130(1):307–17. doi: 10.1007/s10549-011-1605-0

35. Li Y, Hively WP, Varmus HE. Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer. *Oncogene* (2000) 19(8):1002–9. doi: 10.1038/sj.onc.1203273

36. Pond AC, Herschkowitz JI, Schwertfeger KL, Welm B, Zhang Y, York B, et al. Fibroblast growth factor receptor signaling dramatically accelerates tumorigenesis and enhances oncoprotein translation in the mouse mammary tumor virus-Wnt-1 mouse model of breast cancer. *Cancer Res* (2010) 70 (12):4868–79. doi: 10.1158/0008-5472.CAN-09-4404

37. Visvader JE, Stingl J. Mammary stem cells and the differentiation hierarchy: current status and perspectives. *Genes Dev* (2014) 28(11):1143–58. doi: 10.1101/gad.242511.114

38. Davis RT, Blake K, Ma D, Gabra MBI, Hernandez GA, Phung AT, et al. Transcriptional diversity and bioenergetic shift in human breast cancer metastasis revealed by single-cell RNA sequencing. *Nat Cell Biol* (2020) 22(3):310–20. doi: 10.1038/s41556-020-0477-0

39. Padmanaban V, Krol I, Suhail Y, Szczerba BM, Aceto N, Bader JS, et al. E-cadherin is required for metastasis in multiple models of breast cancer. *Nature* (2019) 573(7774):439–44. doi: 10.1038/s41586-019-1526-3

40. Wagner KU, McAllister K, Ward T, Davis B, Wiseman R, Hennighausen L. Spatial and temporal expression of the cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Res* (2001) 10(6):545–53. doi: 10.1023/A:1013063514007

41. Albergaria A, Ribeiro AS, Vieira AF, Sousa B, Nobre AR, Seruca R, et al. P-cadherin role in normal breast development and cancer. *Int J Dev Biol* (2011) 55(7-9):811–22. doi: 10.1387/ijdb.113382aa

42. Ribeiro AS, Sousa B, Carreto L, Mendes N, Nobre AR, Ricardo S, et al. P-cadherin functional role is dependent on e-cadherin cellular context: a proof of concept using the breast cancer model. *J Pathol* (2013) 229(5):705–18. doi: 10.1002/path.4143

43. Sridhar S, Rajesh C, Jishnu PV, Jayaram P, Kabekkodu SP. Increased expression of p-cadherin is an indicator of poor prognosis in breast cancer: a systematic review and meta-analysis. *Breast Cancer Res Treat* (2020) 179(2):301–13. doi: 10.1007/s10549-019-05477-5

44. Vieira AF, Paredes J. P-cadherin and the journey to cancer metastasis. *Mol Cancer* (2015) 14:178. doi: 10.1186/s12943-015-0448-4

45. Cheung KJ, Ewald AJ. A collective route to metastasis: Seeding by tumor cell clusters. *Science* (2016) 352(6282):167–9. doi: 10.1126/science.aaf6546

46. Cheung KJ, Gabrielson E, Werb Z, Ewald AJ. Collective invasion in breast cancer requires a conserved basal epithelial program. *Cell* (2013) 155(7):1639–51. doi: 10.1016/j.cell.2013.11.029

47. Cheung KJ, Padmanaban V, Silvestri V, Schipper K, Cohen JD, Fairchild AN, et al. Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters. *Proc Natl Acad Sci U S A* (2016) 113(7): E854–63. doi: 10.1073/pnas.1508541113

48. Yang C, Cao M, Liu Y, He Y, Du Y, Zhang G, et al. Inducible formation of leader cells driven by CD44 switching gives rise to collective invasion and metastases in luminal breast carcinomas. *Oncogene* (2019) 38(46):7113–32. doi: 10.1038/s41388-019-0899-y

49. Khalil AA, Ilina O, Gritsenko PG, Bult P, Span PN, Friedl P. Collective invasion in ductal and lobular breast cancer associates with distant metastasis. *Clin Exp Metastasis* (2017) 34(6-7):421–9. doi: 10.1007/s10585-017-9858-6

50. Dang TT, Esparza MA, Maine EA, Westcott JM, Pearson GW. DeltaNp63alpha promotes breast cancer cell motility through the selective activation of components of the epithelial-to-Mesenchymal transition program. *Cancer Res* (2015) 75(18):3925–35. doi: 10.1158/0008-5472.CAN-14-3363

51. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. *Nat Rev Mol Cell Biol* (2019) 20(2):69–84. doi: 10.1038/s41580-018-0080-4

52. Pearson GW. Control of invasion by epithelial-to-Mesenchymal transition programs during metastasis. *J Clin Med* (2019) 8(5):646. doi: 10.3390/jcm8050646

53. Kroger C, Afeyan A, Mraz J, Eaton EN, Reinhardt F, Khodor YL, et al. Acquisition of a hybrid E/M state is essential for tumorigenicity of basal breast cancer cells. *Proc Natl Acad Sci U S A* (2019) 116(15):7353–62. doi: 10.1073/pnas.1812876116

54. Erdem C, Nagle AM, Casa AJ, Litzenburger BC, Wang YF, Taylor DL, et al. Proteomic screening and lasso regression reveal differential signaling in insulin and insulin-like growth factor I (IGF1) pathways. *Mol Cell Proteomics* (2016) 15 (9):3045–57. doi: 10.1074/mcp.M115.057729

55. Nagle AM, Levine KM, Tasdemir N, Scott JA, Burlbaugh K, Kehm J, et al. Loss of e-cadherin enhances IGF1-IGF1R pathway activation and sensitizes breast cancers to anti-IGF1R/InsR inhibitors. *Clin Cancer Res* (2018) 24(20):5165–77. doi: 10.1158/1078-0432.CCR-18-0279

56. Farabaugh SM, Boone DN, Lee AV. Role of IGF1R in breast cancer subtypes, stemness, and lineage differentiation. *Front endocrinol* (2015) 6:59. doi: 10.3389/fendo.2015.00059

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 14 December 2022
DOI 10.3389/fonc.2022.988968

OPEN ACCESS

EDITED BY
Svasti Haricharan,
Sanford Burnham Prebys Medical Discovery Institute, United States

REVIEWED BY
Petra Den Hollander,
University of Texas MD Anderson Cancer Center, United States
Ines Barone,
University of Calabria, Italy

*CORRESPONDENCE
Daniela Shveid Gerson
daniela.shveid.gerson@gmail.com

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 07 July 2022
ACCEPTED 14 November 2022
PUBLISHED 14 December 2022

CITATION
Shveid Gerson D, Gerson-Cwilich R, Lara Torres CO, Chousleb de Kalach A, Ventura Gallegos JL, Badillo-Garcia LE, Bargalló Rocha JE, Maffuz-Aziz A, Sánchez Forgach ER, Castorena Roji G, Robles Vidal CD, Vargas-Castillo A, Torres N, Tovar AR, Contreras Jarquín M, Gómez Osnaya JT and Zentella-Dehesa A (2022) Establishment of triple-negative breast cancer cells based on BMI: A novel model in the correlation between obesity and breast cancer. *Front. Oncol.* 12:988968. doi: 10.3389/fonc.2022.988968

Establishment of triple-negative breast cancer cells based on BMI: A novel model in the correlation between obesity and breast cancer

Daniela Shveid Gerson[1*], Raquel Gerson-Cwilich[1], Cesar Octavio Lara Torres[2], Alberto Chousleb de Kalach[3], José Luis Ventura Gallegos[4], Luis Ernesto Badillo-Garcia[4], Juan Enrique Bargalló Rocha[1], Antonio Maffuz-Aziz[1], Ernesto Roberto Sánchez Forgach[1], Gerardo Castorena Roji[1], Carlos D. Robles Vidal[1], Ariana Vargas-Castillo[5,6], Nimbe Torres[6], Armando R. Tovar[6], Mariela Contreras Jarquín[4], Jesús Tenahuatzin Gómez Osnaya[4] and Alejandro Zentella-Dehesa[1,5]

[1]Cancer Center, American British Cowdray (ABC) Medical Center, Mexico City, Mexico, [2]Pathology Department, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico City, Mexico, [3]Experimental Surgery Department, American British Cowdray (ABC) Medical Center, Mexico City, Mexico, [4]Department of Genomic Medicine and Environmental Toxicology, Institute of Biomedical Research, National Autonomous University of Mexico, Mexico City, Mexico, [5]Biochemistry Unit, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico City, Mexico, [6]Department of Nutrition Physiology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico City, Mexico

Introduction: Obesity has been associated with an increased risk of biologically aggressive variants in breast cancer. Women with obesity often have tumors diagnosed at later stages of the disease, associated with a poorer prognosis and a different response to treatment. Human cell lines have been derived from specific subtypes of breast cancer and have served to define the cell physiology of corresponding breast cancer subtypes. However, there are no current cell lines for breast cancer specifically derived from patients with different BMIs. The availability of those breast cancer cell lines should allow to describe and unravel functional alterations linked to these comorbidities.

Methods: Cell cultures were established from tumor explants. Once generated, the triple negative subtype in a patient with obesity and a patient with a normal BMI were chosen for comparison. For cellular characterization, the following assays were conducted: proliferation assays, chemo – sensitivity assays for doxorubicin and paclitaxel, wound healing motility assays, matrix invasion assays, breast cancer

cell growth to estradiol by chronic exposure to leptin, induction of endothelial permeability and tumorigenic potential in athymic mice with normo - versus hypercaloric diets with an evaluation of the epithelium – mesenchymal transformation proteins.

Results: Two different cell lines, were established from patients with breast cancer: DSG-BC1, with a BMI of 21.9 kg/m2 and DSG-BC2, with a BMI of 31.5 kg/m2. In vitro, these two cell lines show differential growth rates, motility, chemosensitivity, vascular permeability, response to leptin with an activation of the JAK2/STAT3/AKT signaling pathway. In vivo, they displayed distinct tumorigenic potential. In particular, DSG-BC2, presented higher tumorigenicity when implanted in mice fed with a hypercaloric diet.

Discussion: To our knowledge, these primary cultures are the first in vitro representation of both breast cancer and obesity. DSG – BC2 presented a more aggressive in vivo and in vitro phenotype. These results support the hypothesis that breast cancer generated in an obese metabolic state may represent a contrasting variant within the same disease. This new model will allow both further comprehension, functional studies and the analysis of altered molecular mechanisms under the comorbidity of obesity and breast cancer.

KEYWORDS

breast cancer, obese adipose tissues, BMI – body mass index, triple negative breast cancer, cell lines, cell culture, leptin and endothelial activation

Introduction

In Mexico, cancer represents the third cause of mortality in women over 40 years of age (1, 2). Among them, breast cancer is the first cause of female mortality associated with neoplastic diseases and hence considered an emergent public health problem (3, 4). This mortality is associated with metastatic invasion of vital organs such as the lung, bone, liver, and brain. Breast cancer incidence and mortality are associated subsequently with two important aspects with regards to the Mexican population: age and overweight. The inversion of the population pyramid predicts an increase in the number of women over 50 years of age in the upcoming decades (5) and an increase in the frequency of obesity and overweight in more than 70% of women in our country (6, 7). In fact, Mexico is considered the country with the highest obesity rates worldwide.

The incidence of breast cancer in obese women is up to three times greater compared to that in women with ideal body weight (8) and is associated with particular biological characteristics. Studies have demonstrated that patients with obesity and cancer have a lower global survival and greater possibility of cancer recurrence. This in turn is caused by hormones involved in obesity, such as adipokines, glucocorticoids, and insulin, secreted in an abnormal fashion and acquire aberrant signaling promoting fat storage (9). This further propagates obesity and an increased production of such hormones, contributing to the development of numerous diseases, among which cancer is highly prevalent. Taking this into consideration, it is important to study whether breast cancer in obese women differs regarding biology and therapeutic susceptibility.

In Mexico, there is but one study that links overweight and obesity as adverse prognostic factors in breast cancer (10, 11). Obesity has been related to multiple cancer subtypes due to the chronic state of inflammation contained in the adipose tissue, the increment in circulating levels of insulin, and the increase in insulin receptors, which favor autonomous growth and alterations in adipokines, hormones, and metabolites associated to epigenetic changes. The systemic changes induced by obesity generate a particular tumorigenic effect, cellular proliferation, cancer progression, and subsequent drug resistance and cancer recurrence (12).

In relation to breast cancer, obesity has been associated with an increased risk of more biologically aggressive variants, notably triple-negative breast cancer. It also promotes invasion and metastasis (13). Women with obesity often have tumors diagnosed at later stages of the disease, which, aside from immuno-phenotype and stage, is thus associated with a poorer prognosis and a different response to treatment.

There are no current cell lines for breast cancer previously derived from patients with different BMI or specifically with an obese phenotype. We aimed to create cell cultures derived from patients with BMI <25and >30 kg/m^2 to further investigate differences between the cellular biology and molecular profiles in breast cancer and obesity compared to breast cancer in women with ideal weight. We established two different cell lines, DSG-BC1 and DSG-BC2, derived from a woman with ideal weight and obesity, respectively, both triple-negative immune subtypes, and performed a preliminary biological characterization. To our knowledge, these primary cultures are the first *in vitro* representation where the influence of the comorbidity of breast cancer and obesity can be compared to breast cancer without overweight or obesity.

Materials and methods

Clinical characteristics

This research protocol was submitted to the research and ethics committee of The ABC Medical Center in Mexico City, approved with registration number ABD 14.04. Inclusion criterion for the study was the following: women with a previous biopsy with a pathological result of breast cancer at any stage, who were subjected to a surgical intervention for the removal of cancer from the breast, in which at least 5 mm^3 of tissue was obtained. Once patients signed the informed consent, they were divided into three groups: BMI below 25 kg/m^2, BMI between 25 and 30 kg/m^2, and those with a BMI over 30 kg/m^2. Exclusion criteria included breast cancer recurrence, inflammatory breast cancer or processes, breast trauma, and previous systemic therapy of any kind in relation to the current breast neoplasm. A total of 32 women participated in the study. They were between 37 and 73 years of age with an average age of 55. Of these women, 27 had ductal invasive breast cancer and 5 with lobular infiltrating carcinomas. Regarding their immune phenotype, there were seven triple-negative breast cancers; the rest were hormone positive, including four triple positives with an HER2 amplification. Six women were obese, 13 were overweight, and 13 had a BMI under 25 kg/m^2. There were 13 patients who presented comorbidities including diabetes mellitus type II (4), hypertension (2), hypothyroidism (3), hypercholesterolemia (1), hiatal hernia (1), gastroesophageal reflux disease (1), and fibromyalgia (1).

Establishment of cell cultures

Patients were selected from the ABC Medical Center Cancer Center, and signature of the informed consent was obtained. Through a collaboration sub-protocol with surgical oncology, nursing, and pathology, we were able to receive tumor fragments of at least 5 mm^3 from excised breast tissue derived from partial or total mastectomies. The tissue fragments were then placed in growth medium for transportation. Once in the sterile Hood, the medium was aspirated, and the explant was placed on sterile gauze to remove excess liquid. The explant was then transferred to a Petri dish where they were cut into approximately 1 mm × 1 mm pieces and spread throughout the dish. Finally, they were attached to the bottom of a 3-cm diameter Petri dish and left to dry for 5 min to assure firm adhesion. After this, 3 ml of growth medium was added, and micrographs of the attached explants were taken with a Primovert inverted microscope (Zeiss). This first image served to keep record of cellular sprouting and growth. Finally, the plates were placed inside an incubator at 37°C under an atmosphere with 5% CO_2 and 100% humidity. Cell sprouting and growth were monitored daily. The first cells to spread and proliferate from the explants were fibroblasts with rapid proliferation rates. After approximately 3–6 weeks, tumor cells began to spread from the periphery of the explants. For isolation of these tumor cells, different procedures were evaluated, including the use of collagenase, manual scraping of fibroblast under the dissection microscope based on cell morphology, differential sedimentation rates, and selection of cellular populations using cloning rings. Manual separation and the selection of clonal populations were the most effective in isolating tumor cells and were the sole method used after the initial 10 explants (Figure 1). A total of 32 tumor explants were obtained. They were paired by histology and immunohistochemistry subtype and matched to have a pair with and without obesity. We were able to establish two cell cultures from patients with ductal invasive triple-negative breast cancer, one without obesity (DSG-BC1) and one with obesity (DSG-BC2).

Proliferation assays

For direct cell count, 12,000 cell/well were plated in 48-well plates (Corning Costar), and every 24 h, the cells of three wells were detached with a Trypsin (0.25%)/EDTA (0.02%) solution in phosphate-buffered saline (PBS) (T4049 Sigma Aldrich, St. Louis, MO) and counted in a hemocytomer chamber by loading the two chambers with a 1:1 mixture v/v of the cell suspension with trypan blue solution. For indirect evaluation by measuring the increase in impedance, proliferation was evaluated indirectly by measuring electric impedance using the xCELLigence system. We plated 7,500 cells/well in a final volume of 200 µl/well in xCelligence 16-well E-plates (Cat No. 5469830001, Aligent, Santa Clara, CA). Every cell line was plated in triplicates. Impedance was measured every 30 min throughout 96 h. The cell division rate was calculated using the software provided with the xCelligence equipment and expressed as change in Cell Index/h.

Chemo-sensitivity assays

Both breast cancer cell lines (DSG-BC-1 and DSG-BC-2) were plated in 48-well plates (Corning Costar) at a cell density of 12,000 cells/well. Three wells with cells remained without treatment, three other wells remained without cells, and all wells contained 500 µl growth medium. After 24 h, cells were exposed to increasing doses of paclitaxel or doxorubicin; 48 h later, the medium was removed by aspiration, and the remaining viable cells were fixed with 2% glutaraldehyde in growth medium for 30 min. The glutaraldehyde solution was removed, and all wells were stained with 250 µl of a solution with 0.5% crystal violet (Sigma Aldrich C0775, St. Louis, MO) in 20% methanol and 80% H_2O v/v for 15 min, and excess satin was rinsed with tap water and allowed the plates to dry under air current. Crystal violet with 10% acetic acid solution was solubilized and read OD at 570 nm. The OD of the wells with cells was subtracted from the rest of the OD values. OD of the wells with cells but without chemotherapy served to assign 100% viability; the rest of the OD values were normalized and expressed as % viability.

Wound healing motility assays

DSG-BC1 and DSG-BC2 cells were plated in 100 × 15 mm Petri dishes (Corning Costar) previously marked with lines in the exterior of the lower part of the Petri dish to guide the scratching. Cells were plated with growth medium at a cell density of 40,000 cells/cm^2. The monolayers reached confluence within 24 h. The monolayers were scratched using a sterile pipette to generate a linear wound in eight different lines/plate. Cell motility was evaluated every 3 h; micrographs were taken for the eight scratched lines for each cell type. Using the image processing software Image J (public domain, Wayne Rasband NIH), the cell-free areas were outlined, and the surface was estimated in pixel units. Cell motility was evaluated as the reduction in cell-free area every 3 h and reported as average and standard deviation.

Sensitization to E2 through chronic exposure to leptin

Sensitization to E2 was performed as previously described (14). Cells (2×10^3) of the different breast cancer cell lines were plated and grown in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% fetal bovine serum (FBS) and leptin (100 ng/ml). All the cell lines were grown at 37°C under a 5% CO_2 atmosphere saturated with 100% H_2O. After 4 days, the medium was replaced with phenol red-free RPMI-1640 supplemented with 10% delipidated FBS and leptin (100 ng/ml) for 24 h. Finally, the medium was replaced with phenol red-free RPMI-1640 supplemented with 5% delipidated FBS and leptin (100 ng/ml); cells were immediately challenged with 0, 10, and 100 nM E2. After 48 h of E2 treatment, cells were fixed and stained with crystal violet as described for the chemo-sensitivity assay.

Preparation of tumor-cell-conditioned medium

Tumor-cell-conditioned medium was prepared as previously described (15). Briefly, each breast cancer cell line was cultured in 20 × 100 mm Petri dishes until it reached 80% confluence. After washing each plate 10 times with 10 ml of PBS/RPMI-1640 (1:1 v/v) without phenol red (Laboratorios Microlab S.A. de C.V., D.F. Mexico, Mexico), the cells were maintained in 8 ml of serum-free RPMI without phenol red per plate. After 48 h incubation at 37°C under an atmosphere with 5% CO_2 and 100% humidity, the culture medium was collected and lyophilized. The resulting powder was dissolved in distilled sterile water (1/10 of the original volume), dialyzed with a PM-3 Ultrafiltration Membrane (EMD Millipore, Billerica, MA, USA), and sterilized using a 0.22-µm Millex-GS syringe filter unit and supplemented with protease inhibitor cocktail (cOmpleteTM Protease Inhibitor Cocktail; Roche Applied Science, Indianapolis, IN, USA). The protein concentration was determined using the Bradford reagent assay (Bio-Rad, Hercules, CA, USA). The concentrated conditioned medium (CM) was kept at 4°C until use in a final dilution 1:10 v/v in growth medium.

Evaluation of endothelial activation

Primary human endothelial cells derived from umbilical veins (HUVECs) were isolated from the veins of umbilical cords within 48 h of normal births as previously described (16). Briefly, the vein was canalized and perfused with PBS to remove blood clots from within. Once cleaned, it was sealed, and a 0.2% collagenase solution (Roche, cat. no. 103586, Basel SZ) prepared in PBS was infused and sealed. The cord was submerged in PBS at 37°C for 10 min and then gently massaged. Afterwards, one end was opened to recuperate dislodged endothelial cells in 50-ml test tubes, and the veins were perfused five times with 10 ml PBS. The mixture of endothelial cells, red blood cells, lymphocytes, and collagenase was centrifuged, and the pellet was reconstituted in endothelial growth medium: M199 medium (Thermo Fisher/GIBCO 11150067) supplemented with 10% FBS, glutamine (2mM), heparin (1 mg/ml) (Sigma Aldrich, St. Louis, MO), and endothelial mitogen (0.01 µg/ml) (Biomedical Technologies Inc., Stoughton, MA). Cells were plated in Petri dishes (Corning Costar); after 24 h, non-

adherent cells were washed away with PBS, and HUVECs were cultured in endothelial growth medium and expanded for the next 2 weeks. Cells were detached from the plate with a Trypsin (0.25%)/EDTA (0.02%) solution in PBS (T4049 Sigma Aldrich, Saint Louis, MO); Endothelial growth medium was then added and centrifuged. The cell pellet was resuspended in endothelial growth medium and counted to plated 50,000 cell/cm^2 for cell permeability assays or for adhesion of U937 cells.

Adhesion of U937 cell to activated endothelial cells

A total of 50,000 HUVECs were plated/well in 24-well plates (Corning Costar) in endothelial growth medium. The cultures reached confluence after 24 h. After treating HUVECs for 3 h with a 1:10 dilution of the concentrated conditioned media from DSG-BC1 or DSG-BC2, the medium was removed, and fresh endothelial growth medium was added. U937 monocytes (1×10^6) were pre-labeled overnight with 1 μCi/ml of 3H-thymidine (NEN, Boston, MA). A total of 250,000 pre-labeled U937 cells were added/well and co-incubated for 3 more hours. At the end of this period, non-adherent cells were removed, and the wells were gently washed with PBS. The radioactivity of the attached cells was counted after cell lysis with 500 μl 0.2 N NaOH and mixed with 3 ml of scintillation fluid (Ultima Gold LLT, Perkin Elmer, Waltham, MA). A triplicate of 250,000 pre-labeled U937 cells were set apart and counted in the scintillation counter. Radioactivity was estimated using a β-Counter (Beckman). The amount of label in adherent cells was normalized using the label of 250,000 pre-labeled cells as 100%.

Changes in endothelial monolayer permeability

For the evaluation of HUVEC monolayer permeability, 12,000 HUVECs cells were plated in a 16-well xCelligence plate and placed in an incubator. When cells reached confluence, an initial reading was conducted to establish a baseline impedance using and RTCADP xCelligence equipment (Agilent, Santa Clara, CA). Afterwards, the conditioned medium was added, and impedance was recorded every 30 min throughout 116 h.

Tumor-cell-conditioned medium

Tumor cells release a variety of bioactive protein factors and metabolites that can be collected in the medium conditioned by the tumor cells. We prepared tumor-cell-conditioned media from DSG-BC1 or DSG-BC2 as previously described (17). Both cell lines were plated at a cell density of 5×10^4 cell/cm^2 in 10-mm Petri dishes with 10 ml DMEM/F12K supplemented with 10% FBS and allowed to reach 80%–90% confluency. At that point, the medium was removed, and the cell monolayers were washed five times with 5 ml PBS to remove excess FBS. The cultures were fed with serum-free DMEM/F12K and incubated for 48 h at 37°C with a 5% CO_2 atmosphere saturated with H_2O. Cell viability was evaluated every 12 h looking for mitotic figures and increase in cell number. After 48 h, the medium was collected and centrifuged at 1,500 rpm for 6 min to remove floating cells. The supernatant was collected and kept 4°C until used in the invasion assay.

Invasion assay

The colorimetric QCM 24-well collagen-based cell invasion assay (Merck ECM508) was used following the vendor's instructions. Briefly, triplicates with 0.25×10^6 cells/0.25 ml of growth medium (DMEM-F12K supplemented with 10% FBS) were seeded in the upper chambers; in the lower chambers, we added 500 μl of DMEM-F12K supplemented with 10% FBS and incubated at 37°C with a 5% CO_2 atmosphere saturated with H_2O. After adhesion for 8 h, the medium in the upper chamber was removed and replaced with growth medium without serum for further 12 h. The assay was started by placing different attractants in the lower chamber: growth medium with 10% FBS, growth medium with 20% FBS, growth medium with phorbol-ester-myristate (PMA) 80 nM final concentration or a 1:1 dilution v/v of serum-free medium conditioned by DSG-BC1 or DSG-BC2 in serum-free growth medium. After 24 h, the cells on both sides of the membrane were fixed and stained following the vendors protocol. Cells from the upper surface of the membrane were removed, and the stain from the cells in the lower surface of the filter was extracted and optical density quantified at 599 nm. OD from the wells with growth medium with 10% FBS was used as controls with 100% invasion.

Soft agar colony formation assay

Colony formation was evaluated using a Nobel-soft system as previously described (18). Briefly, in 12-well plates, a two-layer system with 0.6% and 0.3% agarose content was prepared with 2× serum-free growth medium (DMEM-F12K). The second layer contained 2,500 cells/well. The spaces between the wells were filled with sterile water to reduce desiccation and the plates were incubated at 37°C with a 5% CO_2 atmosphere saturated with H_2O for 3 weeks. Micrographs were taken every 2 days to follow the emergence and growth of 3D colonies. Within the first week, colonies were detected in HeLa cells, which were used in this assay as positive controls.

Cytoplasmic and nuclear extracts

Cytoplasmic and nuclear cell extracts were prepared as previously described (16) from two 10-mm Petri dishes with cell cultures at a confluence of 70%–80%. After scraping the cells in 1 ml PBS, they were subjected to centrifugation at 4,000 rpm for 5 min in a microfuge (Thermo Scientific). The supernatant was removed and the cell pellet frozen by placing the tubes in dry ice with ethanol. Cells were broken by adding 500 µl of a hypotonic buffer [10mM HEPES, pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 1mM dithiothreitol (DTT), and 0.5mM phenylmethylsulfonyl fluoride (PMSF)] and subjected to centrifugation at 4,000 rpm for 5 min. The supernatant was considered to be enriched with cytoplasmic fraction.

Nuclei were gently resuspended in a hypertonic buffer (20mM HEPES, pH 7.9, 400mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, 1mM DTT, and 0.5mM PMSF) and incubated in a rotating mixer (SOL BAT Aparatos Científicos) for 30 min. At the end of this incubation, nuclei were centrifuged at 14,000 rpm for 10 min; the supernatant was considered as the nuclear extract. Both extracts were diluted 1:1 con radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 10mM Tris–HCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.1% Na deoxycholate] supplemented with protease inhibitors (Complete, Roche). Protein concentration was estimated with Bradford reagent in both extracts (BioRad), and equal amounts of protein were diluted in sample buffer to a 1× final concentration (125mM Tris–HCl, pH 6.8, 1% SDS, 10% glycerol, 0.1% bromophenol blue, and 2% 2β-mercaptoethanol).

Western blot

Western blot analysis was performed as previously described (19). Protein content from the cytoplasmic and nuclear extracts were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels, 7.5% acrylamide for cytoplasmic extracts and 10% for nuclear extracts. Once resolved by electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) immobilon-P membranes (Millipore) and blocked with 5% fat-free milk powder in Tris-buffered saline (TBS) overnight at 4°C. After removing the blocking solution and washing the membranes with TBS, primary antibodies (Santa Cruz Biotechnology) were added diluted in TBS overnight at 4°C: anti-E-cadherin 1:250 (sc-8426), anti-vimentin 1:16,000 (sc-6260), anti-Ep-CAM 1:250 (sc-25308), and anti-β-actin 1:10,000 (sc-47778) for the cytoplasmic extracts, and anti-ZEB-1 1:250 (sc-515797), anti-Nanog 1:250 (sc-293121), and anti-PCNA 1:6,000 (sc-56) for nuclear extracts. At the end of the incubation, the primary antibody dilutions were removed, and the membranes were washed for 30 min with TBS-Tween 20, and the secondary antibody anti-murine-HRP (Thermo Scientific) was added for 1 h. At the end, membranes were washed in TBS and developed with Super Signal West Pico plus (Thermo Scientific) following the vendor's instructions. Images were captured using the Fusion Fx Imaging System (Vilber Lourmant) and processed with the Evolution capt software.

Tumorigenic assays

A total of 12×10^6 cells of each of the established cell cultures were resuspended in 100 µl of RPMI-1640 and inoculated into the sub-scapular area of athymic Nu/Nu mice to evaluate for tumorigenesis. Tumor growth was evaluated at 6 weeks, after which the mice were euthanized and the tumor was resected for pathological analysis. These animals had free access to water and standard diet H2916 (Harlan, Indianapolis, IN).

Housing and diet: Normocaloric vs. hypercaloric

The protocol for animal experiments was approved and registered at the research and ethics committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubiran (INCMNSZ) with registration number 1549 (377). Nu/Nu female mice were kept in the animal facility of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán under a protocol approved by the local animal ethics committee. After weaning, the animals were kept under an inverted schedule of 12 h light/12 h darkness in a room at 30°C with free access to water and to AIN-93 normo-caloric diet (20) (Bio-Serv, Flemington NJ) or to an inhouse-prepared high fat/high sugar diet (HFSD) (21). Every 100 g of pelleted diet contained 0.3 g L-cysteine, 0.25 g coline bitartrate, 1 g vitamins, 5 g cellulose, 3.5 g minerals, soy oil, 9 g starch, 11.4 g maltodextrin, 21.3 g sucrose, 24 g casein, 21.88 g lard, and 0.00133 tert-butylhydroquinone (TDHQ) (22). Animal weight was recorded every other day, and NMR was performed every month. Animals were used for the tumorigenesis assay once they reached a weigh of 20 g. Both groups received an implant of 20×10^6 cells from the DSG-BC2 cell lines as described in tumorigenic assay. After 6 weeks, the animals were euthanized, and a necropsy was performed to recover tumors. C57BL/6 female mice were used for comparison of the effect of the hypercaloric diet (HFSD); these animals were kept under an inverted schedule of 12 h light/12 h darkness in a room at room temperature with free access to water and to AIN-93 normo-caloric diet (Bio-Serv, Flemington NJ) or to an inhouse prepared HFSD.

Evaluation of body mass composition by NMR

Body mass composition was analyzed as previously described using an EchoMRI™ nuclear magnetic resonance system (21). Experimental animals were immobilized in restriction cylinders, and NMR signals were captured according to the vendor's specifications. Animals were briefly exposed to a low-intensity electromagnetic field (0.05 T) for 2 min. Data processing provided lean and fat tissue values in grams; these data could be corrected with the total weight of each animal to express percent body fat or lean mass.

Statistical analysis

The patient sample size was evaluated according to a Bernoulli type assay with the following formula for failure and success: $p \pm z_{\alpha/2} \sqrt{(pq/n)}$, where p was the probability of success. It was established to be 0.75, as 0.25 or 25% of the cell cultures would not develop, corresponding to q. $z_{\alpha/2}$ was the probability that the confidence interval would contain μ, which was 95% or z=0.025. For n, or the number of explants needed, 32 patients were needed to establish a confidence interval of (0.60–0.90), such that 60%–90% of cell cultures would ultimately develop. All *in vitro* assays were conducted in triplicate and repeated at least in two or three independent experiments. Statistical significance in these data was established using Student's t-tests and ANOVA, considering statistical significance with a p-value <0.05 (*) or a p-value <0.01 (**).

Results

Establishment of breast cancer cell lines based on BMI

This study was designed to establish breast cancer cell lines based on patients' BMI to be able to determine if breast cancer that develops in a patient with a BMI of 18–25 kg/m^2 has the same biological properties as the one that develops in a patient with obesity (>30 kg/m^2). Tumor explants were obtained from patients with breast cancer and processed to generate cellular colonies attached to the bottom of tissue culture plates. Different methods were evaluated to determine the best way to generate primary cultures enriched in tumor cells (Figure 1). After surgical tumor removal, the pathologist removed small tumor samples (2–5 mm) and placed them in 50-ml sterile tubes with growth medium. Tumor samples were cut into small sections and attached to the surface of a dry Petri dish as described in *Materials and methods*. In the following 2 weeks, fibroblasts, recognized by their flat extended fusiform morphology, began to sprout out of the explants. Within the next 2 weeks, tumor cell colonies could be detected sprouting out of the explants alongside fibroblast proliferation. Tumor cells presented an ovoid or cuboid morphology with large nuclei, typically with two nucleoli and with filipodia and lamellipodia. We designed a cooperative sub-protocol between surgeons, pathologists, medical oncologists, researchers, and nurses that allowed efficient transfer from the operating room to the tissue culture facility reducing the time from the biopsy procurement to tumor clonal proliferation per explants from 2–3 months to 2–3 weeks.

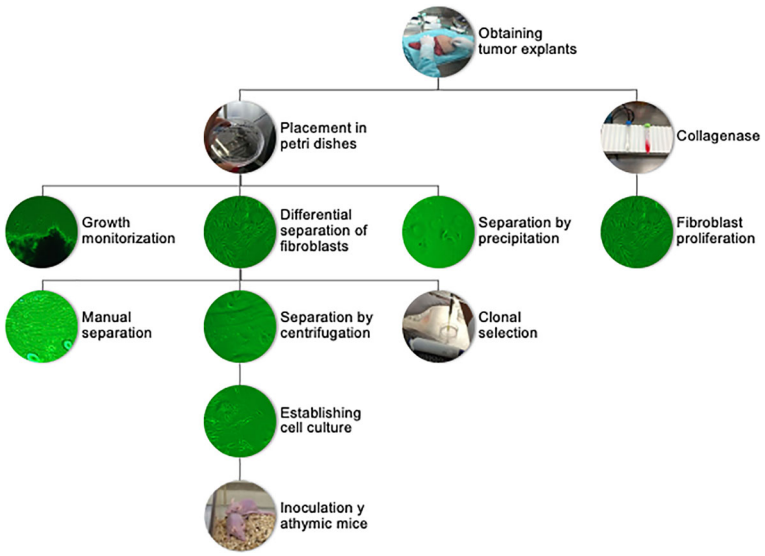


FIGURE 1
Flowchart of sample processing, from tumor explant to primary cultures and tumorigenic assay.

Different methods were tested to obtain cell cultures enriched in tumor cells. The central problem was the elimination of fibroblasts from the primary cultures, which was solved using differential adhesion efficiencies. Cell populations that had emerged from tumor explants were detached using trypsin/EDTA when the Petri dish reached 80% confluence. After centrifugation, the supernatant was removed, and the pellet was resuspended in growth medium. Fibroblasts were allowed to adhere for 20 and 40 min, respectively, and the supernatant enriched in tumor cells was transferred to a new plate. In some instances, we used a cloning ring, specifically when tumor cell colonies grew in areas clearly separated from fibroblasts. To obtain the tumor cell colonies *via* cloning rings, the rings were sealed with silicone grease and placed around the colony. Tumor cells inside the ring were removed using trypsin/EDTA and placed into a separate Petri dish. An alternative approach implied digesting the tumor fragments with type II collagenase (300 U/ml) for 30 min at 37°C, incubating for 20 min in the incubator. The resulting digestion mixture was centrifuged and the pellet resuspended with growth medium and placed in two wells using differential centrifugation of the supernatant at 45, 100, and 200×*g*, respectively. This method was not efficient, as although tumoral fragments were disaggregated, the different cell populations including tumor cells, fibroblasts, epithelial cells, and adipocytes could not be separated. The faster proliferation rate of fibroblasts led to cultures dominated by fibroblasts within 7–10 days. The few tumor cells that were able to proliferate generated isolated colonies. The attempt to remove fibroblasts by scrapping proved to be inefficient. Hence, after adhering a tumor explant and collecting the cell populations that emerged within 2–3 weeks, the differential cell adhesion and ring cloning proved to be the most effective way to generate primary tumor cell cultures free from fibroblasts; *ergo*, they were both utilized in subsequent elaboration of primary tumor cell cultures (23–26).

We were able to establish two triple-negative breast cancer cell lines, one derived from a patient with normal weight (BMI < 25 kg/m^2) and the second from a patient with obesity (BMI > 30 kg/m^2), corresponding to explants 5 and 7. It is important to mention that the explant number 5, from which later the DSG-BC1 cell line was derived, came from a 59-year-old patient with ductal invasive breast cancer and a BMI of 21.9 kg/m^2. Menses began at 14 years and menopause at 47. She had four pregnancies, all resulting in live births. She denied the use of contraception and had only a history of lipoma removal in 2000. The tumor immune phenotype was initially ER+ (92%), PR+ (95%), and HER2-negative tumor. During the late stages of establishing the primary culture, we observed a transformation into a triple-negative phenotype cell culture. With respect to explant number 7, from which later the DSG-BC2 cell line was derived, it came from a 52-year-old patient with invasive ductal carcinoma and a BMI of 31.5 kg/m^2. Menses began at 11 years and menopause at 48. She had two pregnancies, both resulting in spontaneous abortions. She had no comorbidities. The tumor

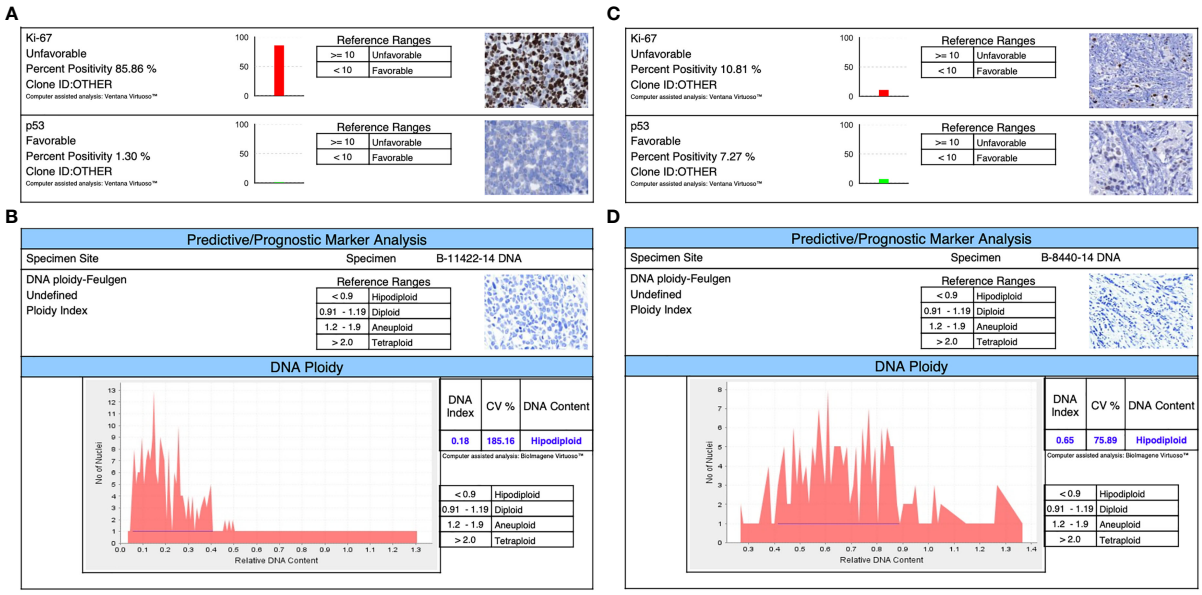


FIGURE 2
Immunophenotyping shows in both triple-negative lines: **(A)** for DSG -BC1, a Ki67 of 85.85% and a TP53 of 1.3%. **(B)** The ploidy of DSG-BC1 revealed a hypodiploid DNA content. **(C)** For DSG -BC2, a Ki67 of 10.81% and a TP53 of 7.27%. **(D)** The ploidy of DSG-BC2 revealed a hypodiploid DNA content.

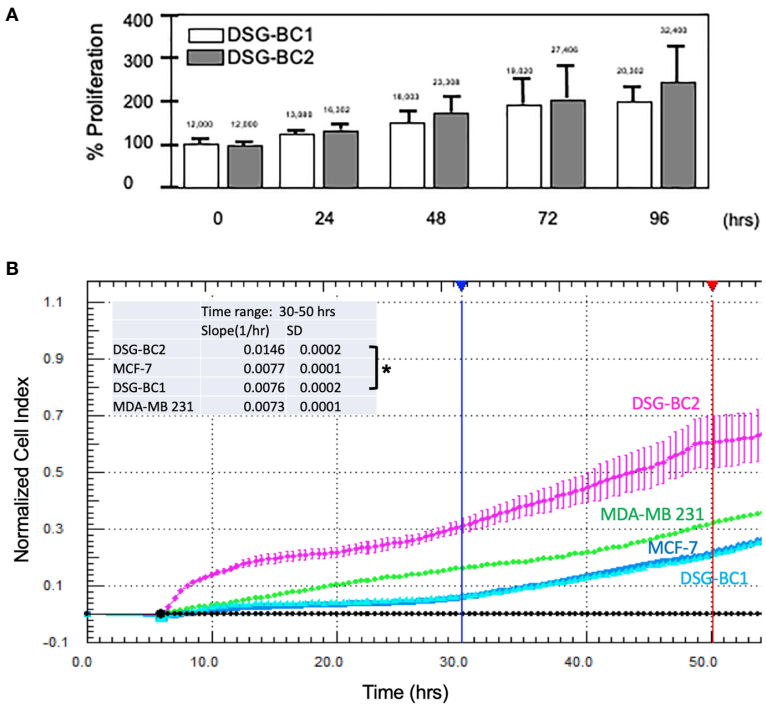


FIGURE 3
Breast cancer cells derived from a patient with obesity display a higher proliferation rate than the one derived from a patient with normal weight. **(A)** Direct cell counts every 24 h. Numbers above each bar represent the average cell number. **(B)** Normalized cell index measured every 30 min between 0 and 5 h. The inset represents the slope (change in normalized cell index/h) between 30 (blue vertical lines) and 50 h (red vertical line) of continuous growth. In panel **(B)**, MCF-7 and MDA-MB-231 cell lines were included as reference in cell growth. Experiment performed in triplicates; values represent average +/– standard deviation of the mean, n = 3. Vertical bracket indicates comparison between the two newly described cell lines, p value < 0.05 (*).

was a triple-negative immune phenotype, and the cell line preserved the same triple negative throughout the study. To be able to correlate findings related to differences in breast cancer in patients with obesity versus normal weight, it was essential to compare tumors with the same immune phenotype. We therefore present two triple-negative cell lines DSG-BC1 (BMI = 21.9) and DSG-BC-2 (BMI = 31.5).

Immunophenotyping

Immunohistochemistry was performed using the Ventana BenchMark Ultra platform (Roche) using the following pre-diluted antibodies: anti-estrogen receptor (clone SP1, rabbit monoclonal, Roche), anti-progesterone receptor (clone 1E2, rabbit monoclonal, Roche), and anti-Her2 (clone 4B5, rabbit monoclonal, Roche). The stained slides were evaluated by a board-certified pathologist with extensive experience on breast pathology and immunohistochemistry using the current ER/PR and Her2 CAP/ASCO guidelines, in relation to the previously mentioned importance of the comparison of tumors with the same immune phenotype. Both cell lines were confirmed as triple negative as seen in Figures 2A–D (27, 28).

Proliferation, migration, and invasion

In an initial proliferation assay, the increase in cell number was analyzed by direct count with a hemocytometer (Figure 3A). Despite having plated the same initial number of cells, DSG-BC2 always related a higher cell count. By 72 h, it had 44% more cells than DSG-BC1. We also evaluated the proliferation rates in early time windows and compared them with the proliferation rates of the MDA-MD-231 and the MCF-7 cell lines. Cell proliferation during the first 50 h after plating was evaluated as an increase in impedance as described in *Materials and methods*. DSG-BC2 was able to grow 1.92 times faster than DSG-BC1 (Figure 3B). On the other hand, DSG-BC1, MCF-7, and MDA-231 presented very similar growth rates (0.077, 0.76, and 0.73 normalized cell index units/h). In both assays, we observed that the cell line DSG-BC2, derived from a patient with high BMI, presented a higher proliferation rate. The proliferation assays conducted lead

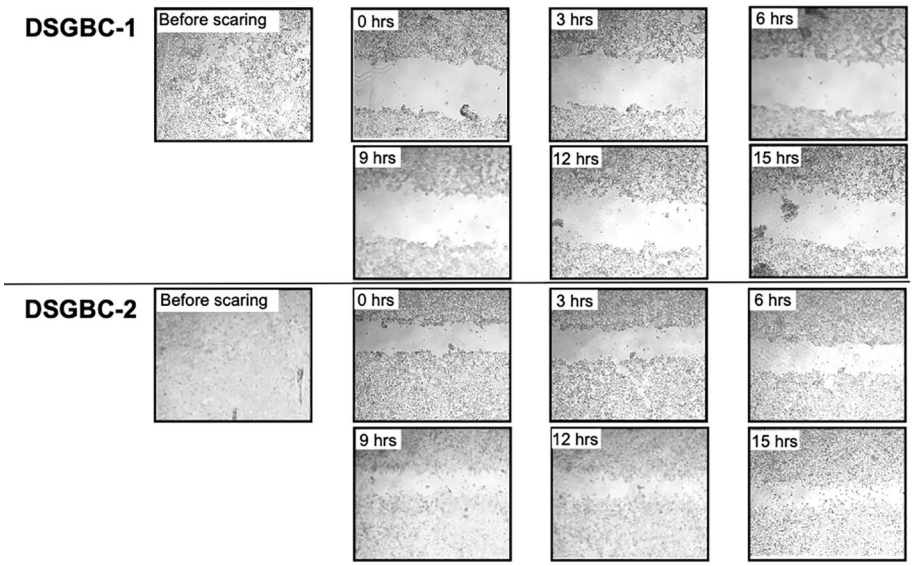


FIGURE 4
Breast cancer cells derived from a patient with obesity are faster in closing the space in a wound healing assay than the one derived from a patient with normal weight. Wound healing assay. Cells were plated to reach confluence within 24 h as described in *Materials and methods*. The invasion of the cell-free area was recorded every 3 h at the indicated time points. The cell-free areas were analyzed by Image J.

TABLE 1 The table presents the average reduction in area (-D Area) every 3h, the bottom line presents the average of the 3 intervals.

	DSGBC1		DSGBC2	
	Speed -Δ Area/3hr		Speed -Δ Area/3hr	
Interval	Avg	Std	Avg	Std
3 to 6 h	10,977	12,877	29,056	17,460
6 to 9 h	20,780	17,811	50,388	30,820
9 to 12 h	25,334	18,659	13,427	12,286
Average interval				
-Δ Area/3h	19,030	3,122	30,957	9,564
Fold	1.00		1.63	

Data represent average +/- standard deviation of the mean, n = 8.

to the understanding that DSG-BC2, the cell line of breast cancer and obesity, showed greater rates of proliferation in the three 2D systems assays in comparison to the cell line without obesity. Although this is seen *in vitro*, it may correlate to aggressive tumor growth *in vivo* in obese patients.

Petri dishes containing confluent cultures of DSG-BC1 and DSG-BC2 were scraped with a sterile pipet tip to create linear "wounds." The separation of the remaining cell edges left a cell-free surface. After 24 h, filipodia and lamellipodia were observed to be emerging from the edges. The wound closing index was estimated with a total close at 72 h for DSG-BC1 and a total closing index at 96 h for DSG-BC2 (data not shown). Since within the timeframe of 72 h cell duplication might contribute to the reduction in free surface, we evaluated the reduction in free surface every 3 h from T0 to T15 hours after generating the linear wounds (Figure 4). The Table 1 shows that the average reduction in free surface was 1.6 times faster in the DSG-BC2 cell line than in the DSG-BC1 cell line. The time frame for wound healing followed every 24 h is consistent with a faster proliferation of DSG-BC2 compared to DSG-BC1, correlating as well to the proliferation assays previously mentioned. The increase in cells in the open space generated when generating the wound follower every 3 h is less likely to be affected by cell division and can be considered to evaluate cellular movement. Hence, the DSG-BC2 breast cancer cell that arose in an obese environment can be seen to "move" faster than its counterpart.

Both DSG-BC1 and DSG-BC2 where exposed to increasing doses of paclitaxel to estimate IC_{50} after 48 h of treatment. The

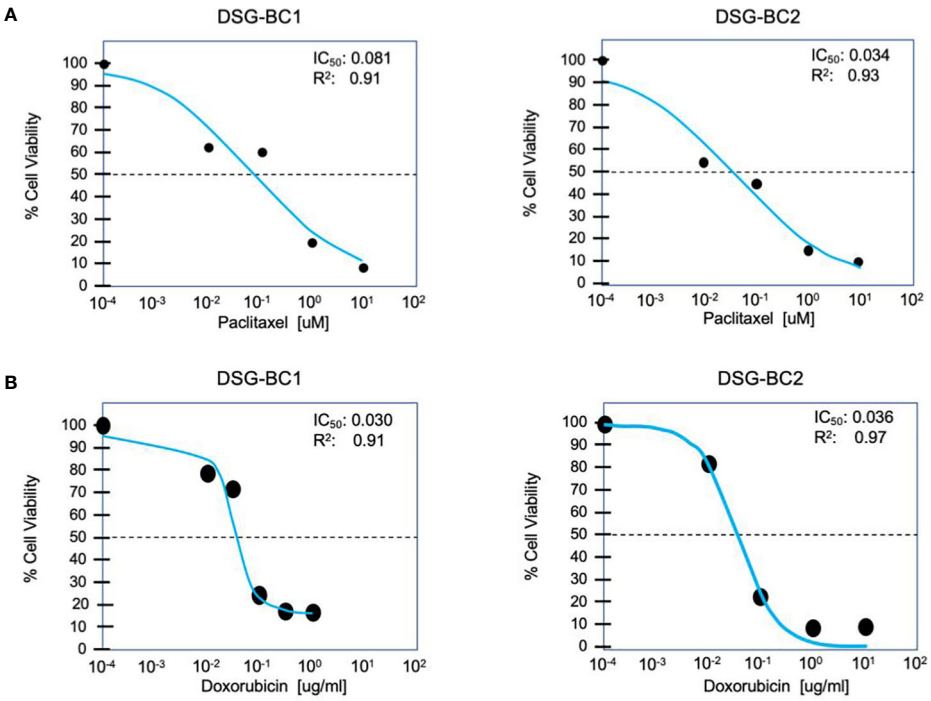


FIGURE 5
Breast cancer cells derived from a patient with obesity display lower chemosensitivity to paclitaxel compared to breast cancer cells derived from a patient with normal weight. Cytotoxicity assay for paclitaxel **(A)** and doxorubicin **(B)** after treatment for 48 h. n = 3.

dose required for IC_{50} in both cell lines was within the same order of magnitude; nevertheless, the cell DSG-BC2 line, derived from the patient with high BMI, required 2.4 times more paclitaxel to reach IC_{50} (Figure 5A). The morphological changes during a time course of 96 h when treated with the IC50 of paclitaxel confirmed approximately 50% cell death after 48 h and 100% cell death after 96 h (Figure 5B). In the same way, both cell lines were exposed to increasing doses of doxorubicin to estimate IC_{50} after 48 h of treatment. In this case, both cell lines had the same IC_{50} of 0.3 µg/ml for doxorubicin (Figure 5B). The cytotoxicity assays for the cell lines show that DSG-BC2 needs higher doses of chemotherapy to be able to achieve similar results in terms of IC_{50} than DSG-BC1. In other words, we observed how breast cancer generated in obesity needed a greater dose of chemotherapy *in vitro* to achieve IC_{50}.

Transformed phenotype and tumorigenic potential of tumor cells correlate with their ability to form 3D colonies when grown in soft agar and with their capacity to invade collagen matrixes. Their commitment in the epithelium–mesenchymal transition (EMT) is also indicative of their aggressiveness. We tested the ability of the DSG-BC1 and DSG-BC2 cell lines to grow by forming 3D colonies in the soft agar using HeLa cells as a positive control. The figure (Figure 6) shows that 2 weeks after initiating the cultures, HeLa cells were able to generate 3D colonies. In contrast, none of the two cell lines DSG-BC1or DSG-BC2 produced any 3D colonies, only isolated groups of a few cells with no indication of mitosis could be detected. The lack of 3D colonies in both cell lines suggests a low tumorigenic potential.

The ability to migrate across the extracellular matrix is an essential hallmark for invasion and metastasis. We used Boyden chambers with 8-µm pores filled with collagen to test the invasive capacity of the two cell lines DSG-BC1and DSG-BC2 using HeLa cells for comparison. Figure 7 shows that both DSG-BC1and DSG-BC2 were able to invade a collagen matrix with a 63% and 42% higher efficiency compared to HeLa cells. The use of 20% FBS in the lower chamber as attractant led to a further increase in invasion in both cell lines: 67% for DSG-BC1and 43% for DSG-BC2 compared to an increase in 238% in HeLa cells. The use of 80 nM PMA in the lower chamber as attractant increased invasion only in DSG-BC1 (47%), while in HeLa cells, PMA increased invasion (234%). In comparison, HeLa cells showed a stronger response to PMA with a 234% increase in invasion. Finally, we also tested a 1:1 dilution of the conditioned media of each cell line (CM) as attractant. CM had no effect on either DSG-BC1and DSG-BC2. These results suggest that both DSG-BC1and DSG-BC2 have the ability to invade collagen matrix, indicative of an aggressive phenotype.

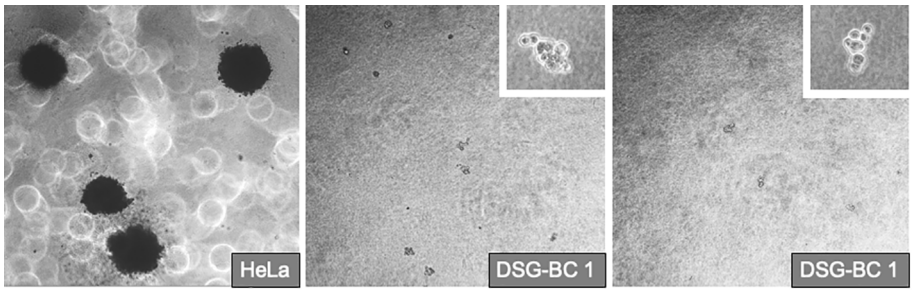


FIGURE 6
Breast cancer cells derived from patients with obesity or normal weight are not able to promote deformation of 3D colonies in soft agar. Even after 2 1/2 weeks after plating in the DSG-BC1 or DSG-BC2 in soft agar no 3D colonies could be detected only isolated cell aggregates could be seen (insets with 100× magnification). In comparison, HeLa cells generated multiple 3D colonies (left image with 40× magnification). n= 6.

Transformation and tumorigenic potential require EMT; therefore, we analyzed the pression of EMT-molecular markers in both DSG-BC1 and DSG-BC2 and in HeLa, MDA-MB-231, and MCF-7 cells used for comparison. Figure 8 shows the cytoplasmic expression of E-cadherin, EPCAM, and vimentin in cytoplasmic extracts and the expression of ZEB1 and Nanog in corresponding nuclear extracts. Both newly established cell lines DSG-BC1 and DSG-BC2 displayed a strong signal for epithelial markers, the cell adhesion molecules E-cadherin and EPCAM. In contrast, the mesenchymal-specific intermediate filament vimentin was absent in both cell lines. Mesenchymal master regulatory transcription factors ZEB1 and Nanog are weakly expressed in the nuclear extracts of both DSG-BC1 and DSG-BC2 cell lines. MCF-7 cells also expressed a strong signal for epithelial markers E-cadherin and EPCAM, but no vimentin. MCF-7 cells displayed a strongest signal for Nanog. In contrast, HeLa and MDA-MB-231 cells displayed a strong expression of vimentin and ZEB1 but had no Nanog expression. The strong expression of epithelial markers in both DSG-BC1 and DSG-BC2 suggests that the EMT is biased towards the epithelial phenotype.

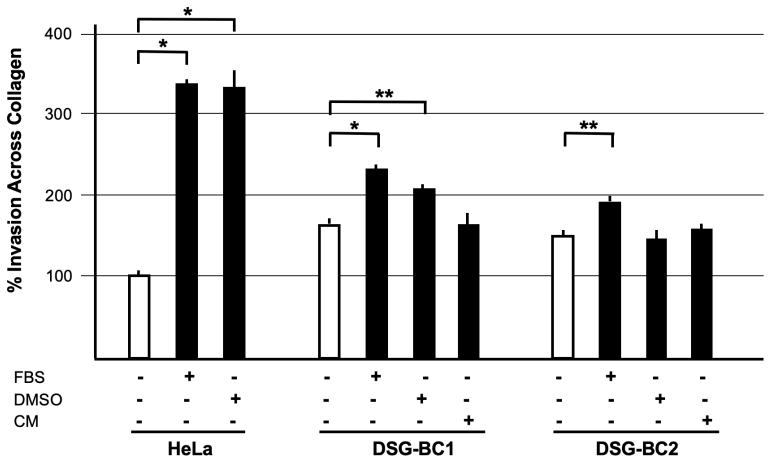


FIGURE 7
Breast cancer cells derived from a patient with obesity presented a higher invasion potential with respect to those derived from patient with normal weight. Invasion assay through collagen filled 8-µm pores. Cells were plated and, after adhesion, starved for 12 h before stimulation with the indicated attractants in the lower chamber as described in *Materials and methods*. HeLa cells were used as a positive control; 10% FBS supplemented growth medium was used as basal attractant in the lower chamber. Invasion of HeLa cells with this condition was considered 100% increase. Positive controls for invasion were 20% FBS (FBS), 80 nM PMA (PMA), or a 1:1 dilution of CM of each cell line. n=3. Brackets indicate comparisons with p value < 0.05 (*) or a p value < 0.01 (**).

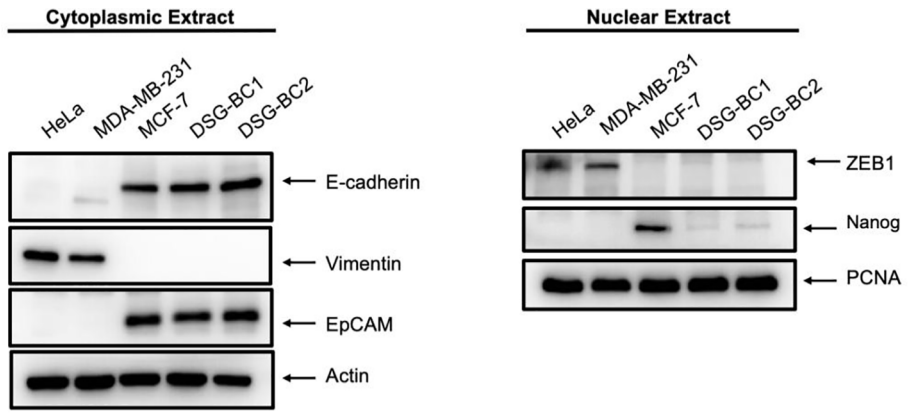


FIGURE 8
Breast cancer cells derived from patients with obesity and normal weight display a strong expression of epithelial markers and a weak and partial expression of mesenchymal markers. Western blot in cytoplasmic (left panel) or nuclear extracts (right panel) were performed as described in materials and methods. 40 ug of total protein were separated using SDS-PAGE and after electro-transfer membranes were probed with the indicated antibodies against EMT markers. Antibodies were diluted as described in material and methods. Actin was used as a loading control for cytoplasmic extract analysis, and PCNA served as loading control for nuclear extracts. Representative image of 3 independent experiments.

Nevertheless, the weak expression of ZEB1 and Nanog suggest that both cell lines have the potential to initiate the EMT.

Endothelial cell activation

Malignancy is related to the secretion of a variety of soluble factors secreted by tumor cells to increase important elements in carcinogenesis, tumor proliferation and metastasis such as increasing vascular permeability and cellular adhesion, phenotypic changes seen in activated endothelial cells. We therefore collected conditioned medium (CM) from DSG-BC1 or DSG-BC2 and processed it as described in *Materials and methods* before testing their effect on the activation of primary human umbilical vein endothelial cells (HUVECs). The CM prepared from DSG-BC1 contained 39.0 µg protein/ml, compared to 19.0 µg protein/ml present in the CM from DSG-BC2. Endothelial cells treated with TNF (10 ng/ml) were able to bind 27 +/− 2% of the added U937 monocytes, while the treatment with CM from DSG-BC1 or DSG-BC2 promoted the adhesion of 18 +/− 3% and 16 +/− 2% of the added U937 cells, respectively (Figure 9A). The binding of the U937 monocytes to the HUVEC endothelial cells aimed to show how the soluble factors released by tumor cells into the CM can lead to an activated endothelial phenotype that could contribute to a metastatic phenotype.

In addition, we also tested the effect of the CM from both cell lines on the permeability of monolayers of HUVECs measuring impedance and so epithelial permeability (Figure 9B). Impedance increased with time in untreated control HUVECs and stabilized 50 h after addition of control medium (red trace in Figure 9B). In the presence of the CM from DSG-BC1, impedance increased in two waves and was markedly reduced 20 h after its addition (blue trace in Figure 9B). In the presence of the CM from DSG-BC2, impedance fell immediately after its addition (green trace in Figure 9B). After 50 h of treatment, the CM from DSG-BC1 had reduced impedance by 20%, while the CM from DSG-BC2 reduced impedance by 52%. Impedance of endothelial cell monolayers is an indirect measure of epithelial permeability known to be affected during metastasis. The reduction in impedance induced by the CM of both cell lines indicates the presence of bioactive secreted products able to reduce endothelial permeability. The fact that the cell line derived from a patient with higher BMI is suggestive of a more aggressive phenotype. With this, an inference on its metastatic potential may be postulated.

Sensitization of breast cancer cell growth to estradiol (E2) by chronic exposure to leptin

Among the different adipokines liberated by adipose tissue in obesity, leptin has been postulated to contribute to breast cancer tumorigenesis and progression. Treating the newly established cell lines DSG-BC-1 and DSG-BC-2 with leptin (100 ng/ml) for 48 h did not affect cell proliferation (data not shown). Leptin is known to have a wide spectrum of biological effects including the sensitization of breast cancer to estradiol-dependent proliferation (14). MCF-7, DSG-BC1, DSG-BC2, and MDA-MB-231 where pretreated with leptin for 5 days and then challenged with 0, 10, and 100 nM E2 for further 48 h. The

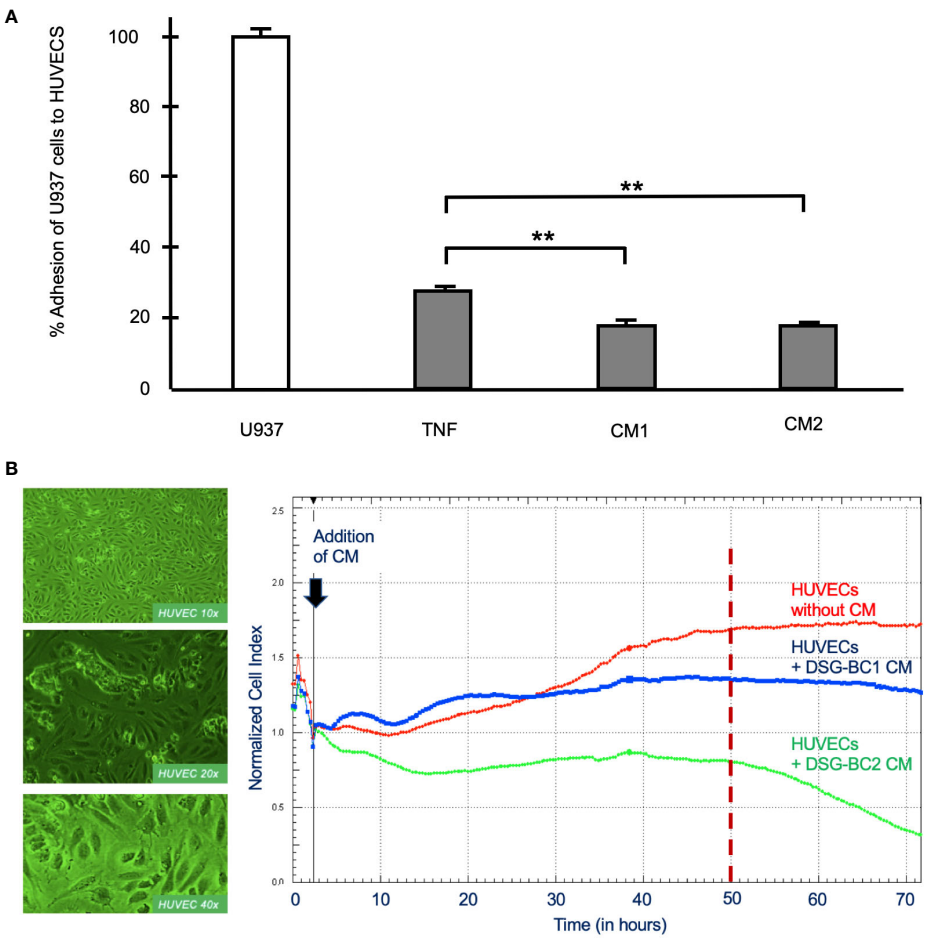


FIGURE 9
Breast cancer cells derived from patients with obesity or normal weight secreted similar amounts of soluble factor capable promoting endothelial cell activation. **(A)** % Adhesion of 3H-thymidine-labeled U937 cells to HUVECs treated for 3 hours with CM from DSG-BC1 or DSG-BC2 as described in materials and methods. 100% corresponds to the amount of radioactivity in 250,000 U937 cells (white bar). Normalized amount of radioactivity bound in HUVECS treated with TNF (10 ng/ml) or with 1:10 dilution of the CM from DSG-BC1 or DSG-BC2 (gray bars), n = 3. Brackets indicate comparisons with p value < 0.05 (*) or a p value < 0.01 (**). **(B)** Left panel: micrographs of endothelial cell monolayers at the indicated magnifications, taken before adding CM. Right panel: representative change in impedance in monolayers of HUVECs treated with a 1:10 dilution of CM of the indicated cell lines. CM were prepared as described in materials and methods and added 48 hours after plating (black arrow). Impedance was measured every 30 minutes as described in materials and methods. CM: conditioned media from the indicated cell type.

histogram in Figure 10 shows that 5-day continuous exposure to leptin sensitized MCF-7 to E2, leading to a 56% increase when treated with 10 nM E2 and a 32% when treated with 100 nM E2. These results are in agreement with previous reports (14). Interestingly, the cell line DSG-BC-2 derived from a patient with obesity presented a 15% increase in proliferation when treated with 100 nM E2, while in DSG-BC-1, breast cancer cells isolated from a patient with normal BMI, pretreated with leptin, led to a 15% decrease in proliferation when stimulated with 100 nM E2. MDA-MB-231 cells displayed no response to E2 or to leptin sensitization. This result suggests that despite the fact that both DSG-BC1 and DSG-BC-2 have a triple-negative phenotype, they express low levels of ER. Our results also indicate that the ER signaling pathway in breast cancer cells derived from an obese patient can be sensitized by leptin, an adipokine normally present in patients with increased dysfunctional adipose tissue.

Leptin activates the JAK2/STAT3/AKT signaling pathway in DSG-BC-1 and DSG-BC-2 breast cancer cells

The sensitization effects suggest that both DSG-BC-1 and DSG-BC-2 cells can respond to leptin. We tested if exposure to a leptin could activate phosphorylation of signaling molecules linked to leptin receptor activation. We treated both DSG-BC-1 and DSG-BC-2 cells and MCF-7 cells with 1,000 ng/ml leptin for 10 and 20 min and evaluated the change in the

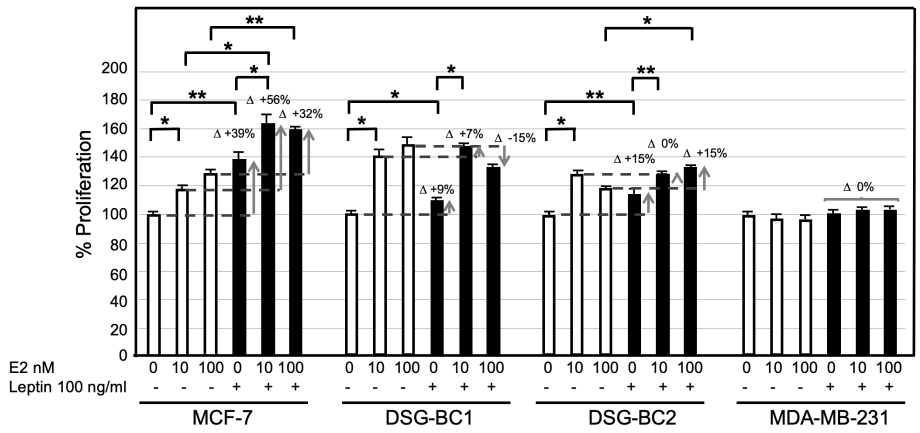


FIGURE 10
Breast cancer cells derived from an obese patient are sensitized to estrogen-proliferation by chronic exposure to leptin. The indicated 4 breast cancer cell lines were cultured as described in materials and methods: treated for 4 days with leptin (100 ng/ml) changed to medium without phenol red and supplemented with lipid free serum for 24 hours before the final stimulation with estradiol (E2) at the indicated concentrations for 48 hrs. Control cultures received no leptin stimulation (empty bars). Sensitization is depicted at the increase in proliferative response (D+). Values are the average of triplicates +/- standard deviation; n = 3. Brackets indicate comparisons with p value < 0.05 (*) or a p value < 0.01 (**).

phosphorylation state of JAK2, STAT3, and AKT through Western blot analysis. Figure 11A shows a representative result of the Western blot analysis, and the histogram in Figure 11B presents the normalized intensity of the phosphorylated signaling proteins. As previously reported, in control MCF-7 cells, leptin induced a transient increase in phosphorylation both of JAK2 and STAT3 in the first 20 min of stimulation, while AKT phosphorylation presented a steady 35% increase. Interestingly the DSG-BC-2 cell line derived from an obese patient displayed a stronger response compared to the DSG-BC-1 cell line derived from a patient with normal BMI. These results strongly suggest that both cell lines present functional leptin receptors and that the JAK2/STAT3/AKT signaling pathway in the cell derived from obese patients presents a stronger response to this adipokine.

In vivo experiments

Seven athymic Nu/Nu female mice, fed with a standard diet, were inoculated with 10×10^6 cells of each cell line, DSG-BC1 and DSG-BC2, totaling 14 mice. Seven days after the inoculation, tumor growth was seen in every mouse. The largest tumor was observed in mouse 1 at 14 days with a 10 × 2 mm tumor on the right subscapular area and mouse 2 at 10 days with a 4 × 5 mm tumor on the right subscapular area, both originating in mice inoculated with the DSG-BC2 cell line (29–31). Immunohistochemical analysis for ER, PR, and HER2 confirmed a triple-negative immune phenotype for both DSG-BC1 and DSG-BC2. The histopathological analysis of the tumors in hematoxylin and eosin-stained slides revealed perivascular, neural, and muscular invasion (Figure 12), which was not observed in comparison to tumors generated by DSG-BC1. Although it was corroborated that both tumors had the same triple-negative immune phenotype, DSG-BC2 showed a greater infiltrative nature with the vascular, neural, and muscular invasion, most likely due to the aggressiveness that the metabolic environment in which it was created confers.

To evaluate the influence of an obesogenic *in vivo* environment, we fed Nu/Nu female mice with a defined normo-caloric rodent diet (AIN93) or a high fat – high sucrose diet (HFGD) as previously described. While HFGD promoted a significant increase in weight in normal C57BL/6 mice, it had a more modest effect in Nu/Nu mice (Figure 13A). Nu/Nu mice fed with the normo-caloric diet AIN93, had on average 5 g lower weight than those fed with HFGD. Despite this difference, no significant change in body mass composition was observed after 3 months (Figure 13A). However, when fed with HFGD, the fat tissue estimated by NMR was higher compared to those fed with AIN93 (Figure 13B). This difference was identified as an increase in subcutaneous fat accumulation. A group of nine Nu/Nu female mice fed with either diet was inoculated with 20×10^6 cells from the DSG-BC2 cell line, and tumor growth was monitored as described in *Materials and methods*. While in the group of mice fed with AIN93, only one mouse developed a tumor mass. In the group fed with HFSD, six animals developed tumor growth (Figure 13B). The unexpected observation that Nu/Nu mice appear to have a higher basal energy expenditure compared to C57BL/6 mice could explain the marginal effect of HFSD in developing overweight. In addition, metabolic analysis of the sera from both groups of Nu/Nu revealed that with AIN93, glucose metabolism was the main source of aerobic metabolism,

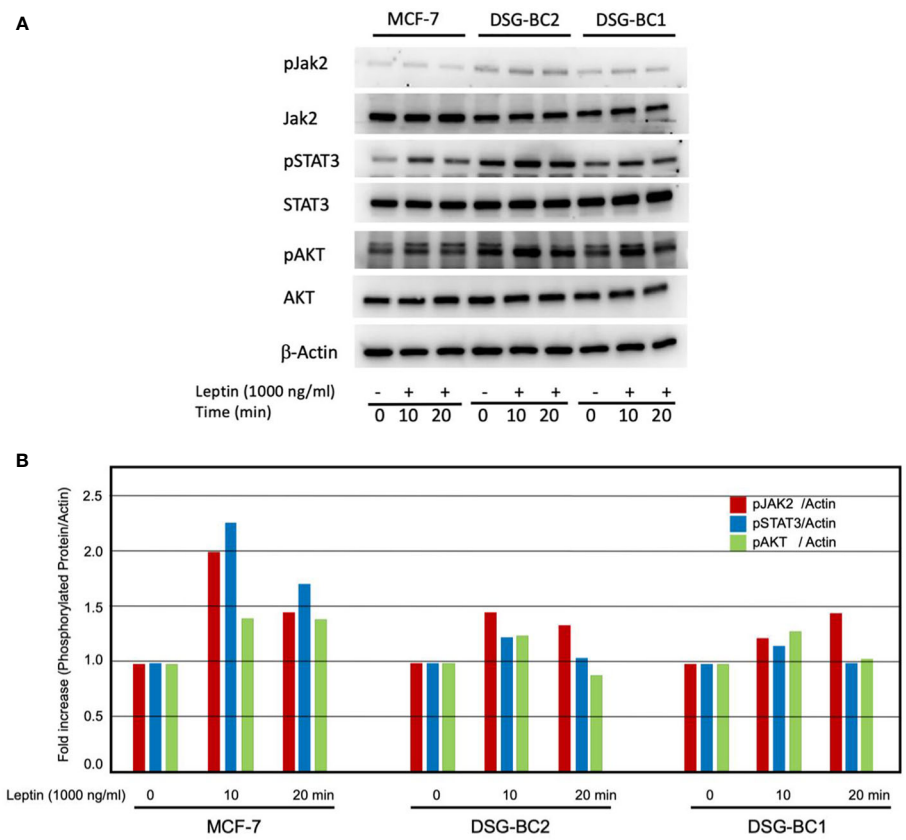


FIGURE 11
Western blot analysis revealed rapid activation of the JAK/STAT3/AKT signaling pathway in response to leptin both in DSG-BC-1 and DSG-BC-2 breast cancer cell lines. Cells (4×10^5) were plated in six-well plates. After 24 h, cell extracts were prepared at the indicated time points (0, 10, and 20 min) of leptin stimulation (1,000 ng/ml), as described in *Materials and methods*. **(A)** Total cell extract (30 µg) was separated in 7.5% SDS-PAGE. Western blot analysis for total JAK2 and phosphorylated JAK2 (pJAK2), total STAT3 and phosphorylated STAT3 (pSTAT3), and total AKT and phosphorylated AKT (pAKT). **(B)** Histogram of the normalized signals of the indicated phosphoproteins of the panel **(A)** Phosphoprotein signals were corrected with the signal of beta actin (β-actin); images were digitalized and analyzed using the public ImageJ software.

while ketone body metabolism was the primary source of energy with HFSD (data not shown). It is possible that these differences are due, at least in part, to the lack of coat in animals of the Nu/Nu strain. This would explain the large difference in the increase in weight when both strains where fed with the hypercaloric diet HFSD (Figure 13A). The greater tumorigenic ability of DSG-BC2 in mice fed with HFSD could pertain to possible growth requirements from the original tumor, which was generated in a metabolically obese environment.

Discussion

Obesity has become an openly recognized risk factor not only for the development of breast cancer but also its aggressiveness and recurrence (15, 32, 33). Women with breast cancer and obesity have a worse disease-free and overall survival despite appropriate local and systemic therapies (34). The importance of this epidemiological comorbidity in Mexico is relevant worldwide (35).

In addition, systemic chemotherapy is less effective, even when dosed appropriately on the basis of actual weight. A central mechanism by which obesity stimulates cancer progression is through chronic, low-grade inflammation in adipose tissue. Counter measures such as exercise in the AIM trial (36) seek to alleviate and reduce systemic inflammation, metabolic diseases, possibly leading to interrelated biomarkers involved in the associations between obesity, exercise, and breast cancer prognosis. Notwithstanding, we currently are in a discovery phase where factors within obesity, adipocytes, and inflammation factors change longstanding paradigms related to cancer treatment. Mechanisms underlying the obesity–

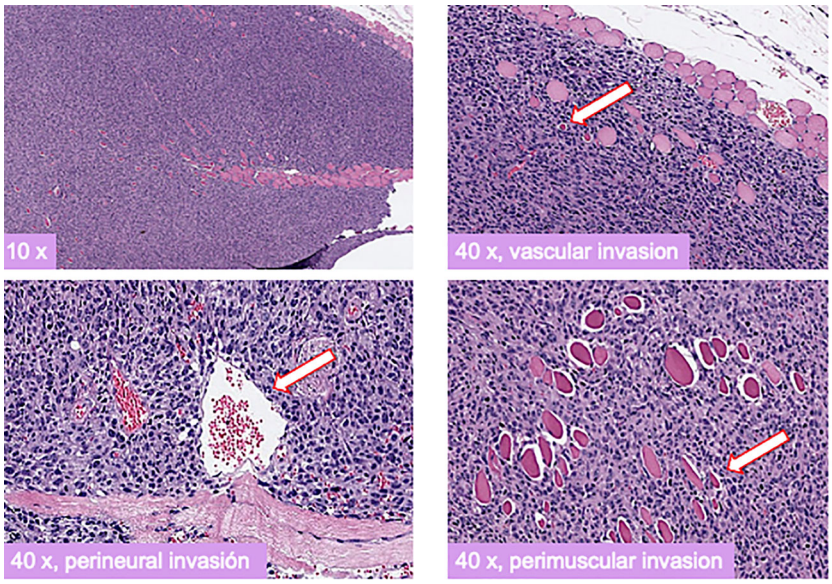


FIGURE 12
Xenotransplants of the breast cancer cells derived from a patient with obesity displayed a more aggressive tumorigenic and invasive activity compared to those derived from a patient with normal weight. Histopathology of H&E-stained slides from tumors generated 3 weeks after implantation of cell from the DSG-BC2 cell line. Images were taken at the indicated magnifications. White arrows indicate transversal section of a vascular structure (upper right panel), transversal section of a nerve (lower left panel), and transversal section of a muscle fiber (lower right panel). Samples were processed as described in *Materials and methods*.

cancer relationship are poorly understood (37, 38). Patients who are obese require special treatment considerations for adequate management and optimal therapeutic efficacy (39).

An important element in the connection with breast cancer and the obese setting is the adipose tissue microenvironment with its complex association to inflammatory factors that promote tumor growth, invasion, and metastasis (40). One of the most influential factors is the adipokine leptin, which is an important molecular mediator of the obesity–breast cancer axis. Increase adiposity, and thus increased leptin secretion, promotes tumor cell proliferation as an independent factor for neoplastic aggressiveness, with functions strengthened through interactions with multiple oncogenes, growth factors, and cytokines (41, 42). It has even been proposed as a novel target in therapeutic strategies for breast cancer given the rise in immune therapy and its proinflammatory mediation (43, 44). The evaluation of leptin levels among the two cell lines is an important step needed in their further characterization.

The link between obesity and breast cancer is clearly a multifactorial and dynamic process that includes pre- versus post-menopausal condition, relative abundance of visceral versus subdermic adipose tissue, and ethnicity (45–47). In post-menopausal women, adipokines also contribute to the risk of breast cancer. While circulating levels of leptin have a strong direct correlation to the incidence of breast cancer, high levels of adiponectin have a protective effect (48, 49). This altered adipokine balance has been postulated to drive the increased expression of aromatase in obese adipose stromal cells leading to an increase in biotransformation of androgenic substrates into estrogens (49, 50), and coincides with higher incidence of ER+ breast cancer in obese patients (46, 48). Other hallmarks of malignancy such as motility, invasion, and anti-tumor immunity are promoted by cancer-associated adipocytes (51, 52). In breast cancer linked to obesity, the non-genomic estrogen receptor crosstalk with the PI3K/Akt and MAPK pathways is increased (53), and the genomic methylation state is altered and related to survival expectancy (54). The response to leptin presented in Figures 6, 7 indicates that both cell lines have leptin receptors, although specific proof is still necessary by Western analysis and RT-PCR. In particular, activation of the JAK2/STAT3/AKT signaling pathway further indicates the functionality of these receptors. The fact that a more robust phosphorylation could be observed in DSG-BC-2 derived from an obese patient is in agreement with the postulated role of leptin in the promotion of breast cancer cells in obese patients. The sensitization to E2 is particularly interesting, since it could reveal a crosstalk between ER expression, ER signaling and leptin signaling. Induction of functional ERs has been reported in human breast cancer cells, promoting conversion of ER− to ER+ cells amenable to antiestrogen therapy (55, 56). Nevertheless, a more careful analysis of the signaling events and changes in gene expression must be performed to confirm this view.

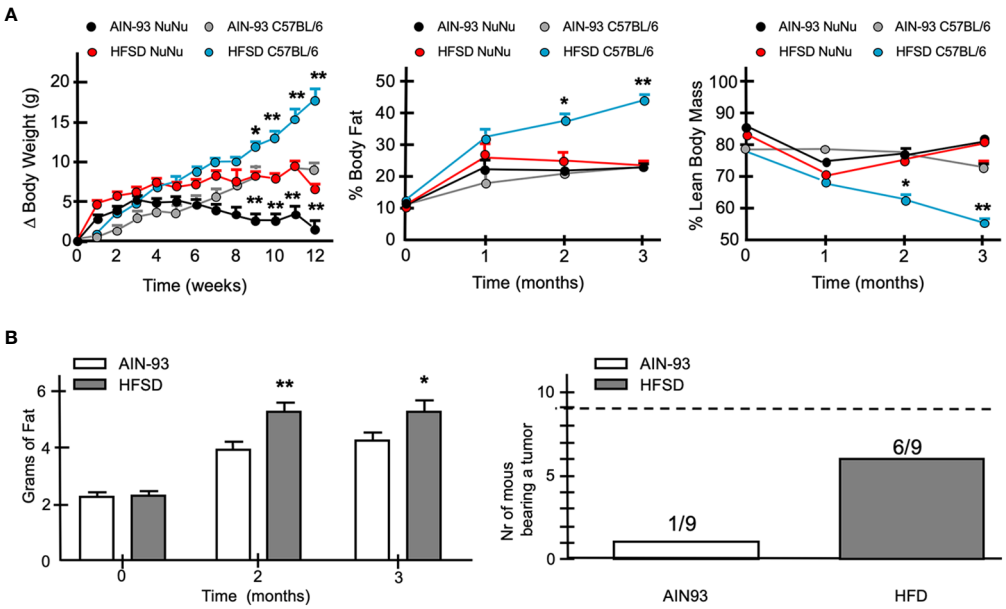


FIGURE 13
When fed a hypercaloric diet Nu/Nu female mice mimic a mild overweight condition and favor more frequent tumor development of DSG-BC-2 xenotransplants. Tumorigenesis in female Nu/Nu mice fed with normocaloric (AIN93) o hypercaloric (HFSD) diet from weaning to the end of the tumorigenic assay. Animals were kept as described in materials and methods until they reached a weight of 20 g. **(A)** Increase in weight once they reached 20 g in Nu/Nu or C57B/6 female mice receiving AIN93 or HFSD (left panel), % of body fat (central panel) and % lean body mass (right panel). **(B)** Total weight estimated by NMR of Nu/Nu mice fed with the AIN93 or HFSD (left panel). Number of the mice bearing tumors from a total of 9 mice that received 20 x 106 cell of the DSG-BC2 that were fed with either AIN93 or HFSD (right panel); n = 9, p value < 0.05 (*) or a p value < 0.01 (**).

Human cell lines have been derived from specific subtypes of breast cancer and have served to define the cell physiology of the corresponding breast cancer subtypes (57). The luminal-A MCF-7 cell line, established in 1973 at the Michigan Cancer Foundation, was derived from a pleural effusion from a 69-year-old white patient with recurrent disease (26, 58). Through functional assays model, cell lines such as MCF-7 cells have led to the identification of a complex epigenetic mechanism where a PAD2-dependent histone H3R26 citrullination facilitates ER transcription activation (59). This cell line has also served to identify the participation of miR23a in the induction of EMT and metastasis in luminal A breast cancer (60). The availability of breast cancer cell lines derived from patients with defined BMI and metabolic alterations related to overweight and obesity will allow to describe and unravel functional alterations linked to these comorbidities (61–63). The triple-negative MDA-MB-231 cell line was derived from a pleural effusion from a 51-year-old Caucasian woman with a metastatic mammary adenocarcinoma with a marked increase in chromosome number, between 65 and 69 (64); nevertheless, no information is available.

Starting with solid tumor biopsies and using standard explant cell culture techniques combined with differential cell adhesion and ring cloning, we established two cell lines DSG-BC1 and DSG-BC2 that display a triple-negative molecular phenotype. DSG-BC1 was derived from a 59-year-old patient with ductal invasive breast cancer and a BMI of 21.9 kg/m^2. DSG-BC2 was derived from a 52-year-old patient with invasive ductal carcinoma and a BMI of 31.5 kg/m^2.

A comparison of basic biological characteristics revealed differences in their proliferating potential and in their ability to move into a cell-free space in a wound healing assay. They presented minor differences in their sensitivity to paclitaxel but were equally sensitive to doxorubicin. Their secretoma was able to activate endothelial cells inducing similar pro-adhesive phenotypes but displayed a different ability to increase endothelial permeability. The behavior of all these *in vitro* assays of the DSG-BC2 cell line suggests a more aggressive phenotype with a faster growth rate and motility, lower sensitivity to paclitaxel, and higher ability to induce endothelial permeability. Even more, in the tumorigenic assay in Nu/Nu mice fed with a normo-caloric diet, the DSG-BC2 cell line presented vascular, neural, and muscular invasion compared with the DSG-BC1 cell line. Nevertheless, the most significant difference was their tumorigenic ability when implanted in Nu/Nu mice fed with diets with different caloric content, trying to mimic eutrophic versus adipogenic *in vivo* metabolic conditions. The DSG-BC2 cell line generated tumors with higher frequency

(6/9) when implanted in mice fed with a hypercaloric diet (HFSD) compared to a lower frequency (1/9) in mice fed with the normocaloric diet (AIN93). This result suggests that the obese condition of the patient where this tumor developed left growth requirements imprinted that favored tumor growth in the obesogenic environment created by the hypercaloric diet.

Taken together, the proliferation potential, motility, *in vitro* invasive capacity, the expression of mesenchymal markers, and *in vivo* tumorigenicity and invasive capacity suggest that tumor cells generated in an obese individual are more aggressive than their counterparts that arise in patients with normal weight.

Conclusion

We present a novel approach to study the comorbidity of obesity and breast cancer by establishing cell lines derived from patients with breast cancer and with different BMIs. We also report that in addition to differences in the biological characteristic analyzed *in vitro*, these cells present differences in their tumorigenic capacity when implanted in mice fed with normo- versus hyper-caloric diets. This new model will allow functional studies and the analysis of altered molecular mechanisms under the comorbidity of obesity and breast cancer.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by research and ethics committee, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Author contributions

DSG, RG-C, AC, and AZ-D contributed to conception and design of the study. DSG and AZ-D wrote sections of the manuscript. CL performed the pathological analysis on the original tumor explants and the mice specimens and wrote sections of the manuscript. JB, AM-A, ES, GC, and CR are the oncologic surgeons involved in patient selection and history, as well as obtaining tumor explants. JV contributed to the establishment of the cell lines. AV, NT, AT, LBG, MC, and JG contributed with the evaluation of obesity in the cell lines, contributed with the evaluation of the cell lines in obese mouse models, designed the high fat and sugar diets, wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

Funding was provided by the Peripheral Unit of the Institute for Biomedical Sciences, UNAM and the Unit of Biochemistry of the INCMNSZ. Proposal No 733 from the Frontiers of Science Program CONACYT and The Department of Oncology.

Acknowledgments

The authors thank the participation of The American British Cowdray Medical Center and Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ)), and the National Autonomous University of Mexico (UNAM) and its peripheral science unit. We would like to give special thanks to: Dra. Vanessa Fuchs Tarlovsky, Dra. Andrea Ballesteros, Dr. Abigail S. Mateos Soria, Dr. Eduardo Torres Cisneros, Alberto Cabrera, Raquel Toquiantzi, Martin Gallardo, Ileana Fernández, and Javier Hernández, Dr. Alberto Villalobos Prieto, Dr. José Fabián Martínez Herrera, Dra. Diana Alejandra Villegas Osorno, Dr. Moisés Mercado Atri, Dr. Ervin Saul Enciso López, Dr. Efraín Isaías Camarín Sánchez, Dr. Alejandro Noguez Ramos, Dra. Lorena López Zepeda.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. INEGI. Principales causas de mortalidad por residencia habitual, grupos de edad y sexo del fallecido. In: *Mortalidad*. México: INEGI (2020). Available at: https://www.inegi.org.mx/app/tabulados/interactivos/?pxq=Mortalidad_Mortalidad_01_de39bf93-dabf-4259-b7d9-990e7b7611bf&idrt=127&opc=t.

2. Romero Figueroa MS, Santillán Arreygue L, Olvera Hernández PO, Morales Sánchez MA, Ramírez Mendiola VL. Frecuencia de factores de riesgo de cáncer de mama. *Ginecol Obstet Mex.* (2008) 76(11):667–72.

3. Diario Oficial de la Federación [DOF]. Norma Oficial mexicana NOM-041-SSA2- 2011. In: *Para la prevención, diagnóstico, tratamiento, control y vigilancia epidemiológica del cáncer de mama*. México: Diario Oficial de la Federación (2011). Available at: http://dof.gob.mx/nota_detalle.php?codigo=5194157&fecha=09/06/2011.

4. Cárdenas Sánchez J, Erazo Valle-Solís AA, Arce Salinas C, Bargalló Rocha JE, Bautista Piña V, Cervantes Sánchez MG, et al. Mexican Consensus on breast cancer diagnosis and treatment. *Gaceta Mexicana Oncología.* (2021) 20(Suppl 2):1–4. doi: 10.24875/j.gamo.M21000215

5. Herschkowitz JI. *Breast cancer subtypes, mouse models, and microarrays*. North Carolina, EUA: University of North Carolina at Chapel Hill (2007). Available at: https://cdr.lib.unc.edu/indexablecontent/uuid:2c573582-6062-46b6-95f8-02594590bfa0. Degree of Doctor of Philosophy in the Curriculum of Genetics and Molecular Biology.

6. Iorns E, Drews – Elger K, Ward TM, Dean S, Clarke J, Berry D, et al. A new mouse model for the study of human breast cancer metastasis. *PloS One* (2012) 7 (10):e47995. doi: 10.1371/journal.pone.0047995

7. Barquera S, Hernández-Barrera L, Trejo-Valdivia B, Shamah T, Campos-Nonato I, Rivera-Dommarco J. Obesity in Mexico, prevalence and trends in adults. *ENSANUT 2018-19.Salud Publica Mex.* (2020) 62:682–92. doi: 10.21149/11630

8. Calle EE, Thun MJ. Obesity and cancer. *Oncogene* (2004) 23(38):6365–78. doi: 10.1038/sj.onc.1207751

9. Aguilar Cordero MJ, González Jiménez E, García López AP, Álvarez Ferré J, Padilla López CA, Guisado Barrilao R, et al. Obesidad y su implicación en el cáncer de mama. *Nutrición Hospitalaria.* (2011) 26:899–903.

10. Arce-Salinas C, Aguilar-Ponce JL, Villarreal-Garza C, Lara-Medina FU, Olvera-Caraza D, Alvarado Miranda A, et al. Overweight and obesity as poor prognostic factors in locally advanced breast cancer patients. *Breast Cancer Res Treat* (2014) 146(1):183–8. doi: 10.1007/s10549-014-2977-8

11. Herrera LA, Mohar Betancourt A. La investigación traslacional en cáncer: Reto del instituto nacional de cancerología. *Rev Investigación Clínica* (2009) 61 (6):451–453.

12. Park J, Morley TS, Kim M, Clegg DJ, Scherer PE. Obesity and cancer–mechanisms underlying tumour progression and recurrence. *Nat Rev Endocrinol* (2014) 10(8):455–65. doi: 10.1038/nrendo.2014.94

13. Rose DP, Gracheck PJ, Vona-Davis L. The interactions of obesity, inflammation and insulin resistance in breast cancer. *Cancers (Basel).* (2015) 7 (4):2147–68. doi: 10.3390/cancers7040883

14. Valle A, Sastre Serra J, Oliver J, Roca P. Chronic leptin treatment sensitizes MCF-7 breast cancer cells to estrogen. *Cell Physiol Biochem* (2011) 28(5):823 –32. doi: 10.1159/000335796

15. Lee K, Kruper L, Dieli-Conwright CM, Mortimer JE. The impact of obesity on breast cancer diagnosis and treatment. *Curr Oncol Rep* (2019) 21(5):41. doi: 10.1007/s11912-019-0787-1

16. MejíaRangel J, Córdova E, Orozco L, VenturaGallegos JL, MitreAguilar I, EscalonaGuzmán A, et al. Pro-adhesive phenotype of normal endothelial cells responding to metastatic breast cancer cell conditioned medium is linked to NFκB-mediated transcriptomic regulation. *Int J Oncol;* (2016) 49:2173–85. doi: 10.3892/ijo.2016.3705

17. Montes-Sánchez D, Ventura JL, Mitre I, Frías S, Michán L, Espejel-Nuñez A, et al. Glycosylated VCAM-1 isoforms revealed in 2D western blots of HUVECs treated with tumoral soluble factors of breast cancer cells. *BMC Chem Biol* (2009) 9:7. doi: 10.1186/1472-6769-9-7

18. Borowicz S, Van Scoyk M, Avasarala S, Karuppusamy Rathinam MK, Tauler J, Bikkavilli RK, et al. The soft agar colony formation assay. *J Vis Exp* (2014) 92): e51998. doi: 10.3791/51998

19. Flores-García LC, Ventura-Gallegos JL, Romero-Córdoba SL, Hernández-Juárez AJ, Naranjo-Meneses MA, García-García E, et al. Sera from women with different metabolic and menopause states differentially regulate cell viability and akt activation in a breast cancer *in-vitro* model. *PloS One* (2022) 17(4):e0266073. doi: 10.1371/journal.pone.0266073

20. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 Purified Diets for Laboratory Rodents: Final Report of The American Institute of Nutrition Ad Hoc Writing Committee on The Reformulation of The AIN-76A Rodent Diet. *J Nutr* (1993) 123:1939–51.

21. Vargas-Castillo A, Tobon-Cornejo S, Del Valle-Mondragon L, Torre-Villalvazo I, Schcolnik-Cabrera A, Guevara-Cruz M, et al. Angiotensin-(1-7) induces beige fat thermogenesis through the mas receptor. *Metabolism* (2020) 103:154048. doi: 10.1016/j.metabol.2019.154048

22. Hsieh CH, Rau CS, Wu SC, Yang JC, Wu YC, Lu TH, et al. Weight-reduction through a low-fat diet causes differential expression of circulating microRNAs in obese C57BL/6 mice. *BMC Genomics* (2015) 16(1):699. doi: 10.1186/s12864-015-1896-3

23. Bana E, Bagrel D. *In vitro* breast cancer models as useful tools in therapeutics? breast cancer - focusing tumor microenvironment, stem cells and metastasis. Croatia. In: *Tech Europe* (Rijeka, Croatia: InTech) (2011). Available at: http://www.intechopen.com/books/breast-cancer-focusing-tumor-microenvironment-stem-cells-and-metastasis/in-vitro-breast-cancer-models-as-useful-tools-in-therapeutics-.

24. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res* (2008) 10(2):R25. doi: 10.1186/bcr1982

25. Lasfargues EY, Ozzello L. Cultivation of human breast carcinomas. *J Natl Cancer Inst* (1958) 21:1131–47.

26. Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* (1973) 51 (5):1409–16. doi: 10.1093/jnci/51.5.1409

27. Allison KH, Hammond MEH, Dowsett M, McKernin SE, Carey LA, Pitzgibbons PL, et al. Estrogen and progesterone receptor testing in breast cancer: ASCO/CAP guideline update. *J Clin Oncol* (2020) 38(12):1346–66. doi: 10.1200/JCO.19.02309

28. Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical Oncology/College of American pathologists clinical practice guideline focused update. *J Clin Oncol* (2018) 36(20):2105–22. doi: 10.1200/JCO.2018.77.8738

29. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. *Breast Cancer Res* (2011) 13(14):215. doi: 10.1186/bcr2889

30. Usary J, Zhao W, Darr D, Roberts PJ, Liu M, Balletta L, et al. Predicting drug responsiveness in human cancers using genetically engineered mice. *Clin Cancer Res* (2013) 19(17):4889–99. doi: 10.1158/1078-0432.CCR-13-0522

31. Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PloS One* (2009) 4(7):e6146. doi: 10.1371/journal.pone.0006146

32. Engmann NJ, Golmakani MK, Miglioretti DL, Sprague BL, Kerlikowske K. Breast cancer surveillance consortium. population-attributable risk proportion of clinical risk factors for breast cancer. *JAMA Oncol* (2017) 3(9):1228–36. doi: 10.1001/jamaoncol.2016.6326

33. Group PBCC, Schoemaker MJ, Nichols HB, Wright LB, Brook MN, Jones ME, et al. Association of body mass index and age with subsequent breast cancer risk in premenopausal women. *JAMA Oncol* (2018) 4(11):e181771. doi: 10.1001/jamaoncol.2018.1771

34. García-Estévez L, Cortés J, Pérez S, Calvo I, Gallegos, Moreno-Bueno G. Obesity and breast cancer: A paradoxical and controversial relationship influenced by menopausal status. *Front Oncol* (2021) 11. doi: 10.3389/fonc.2021.705911

35. Amadou A, Torres Mejia G, Fagherazzi G, Ortega C, Angeles-Llerenas A, Chajes V, et al. Anthropometry, silhouette trajectory, and risk of breast cancer in Mexican women. *Am J Prev Med* (2014) 46(3 Suppl 1):S52–64. doi: 10.1016/j.amepre.2013.10.024

36. Dong-Woo K, Wilson Rebekah L, Gonzalo-Encabo P, Norris MK, Hans M, Tahbaz M, et al. Targeting adiposity and inflammation with movement to improve prognosis in breast cancer survivors (The AIM trial): Rationale, design, and methods. *Front Oncol* (2022) 12:202. doi: 10.3389/fonc.2022.896995

37. Ewertz M, Jensen MB, Gunnarsdóttir KÁ, Højris I, Jakobsen EH, Nielsen D, et al. Effect of obesity on prognosis after early-stage breast cancer. *J Clin Oncol* (2011) 29(1):25–31. doi: 10.1200/JCO.2010.29.7614

38. Garland M, Hsu FC, Clark C, Chiba A, Howard-McNatt M. The impact of obesity on outcomes for patients undergoing mastectomy using the ACS-NSQIP data set. *Breast Cancer Res Treat* (2018) 168(3):723–6. doi: 10.1007/s10549-017-4651-4

39. Quail DF, Dannenberg AJ. The obese adipose tissue microenvironment in cancer development and progression. *Nat Rev Endocrinol* (2019) 15(3):139–54. doi: 10.1038/s41574-018-0126-x

40. Barone I, Giordano C, Bonofiglio D, Andò S, Catalano S. Leptin, obesity and breast cancer: progress to understanding the molecular connections. *Curr Opin Pharmacol* (2016) 31:83–9. doi: 10.1016/j.coph.2016.10.003

41. Strong AL, Ohlstein JF, Biagas BA, Rhodes LV, Pei DT, Tucker HA, et al. Leptin produced by obese adipose stromal/stem cells enhances proliferation and metastasis of estrogen receptor positive breast cancers. *Breast Cancer Res* (2015) 17 (1):112. doi: 10.1186/s13058-015-0622-z

42. Sánchez-Jiménez F, Pérez-Pérez A, de la Cruz-Merino L, Sánchez-Margalet V. Obesity and breast cancer: Role of leptin. *Front Oncol* (2019) 9:596. doi: 10.3389/fonc.2019.00596

43. Atoum MF, Alzoughool F, Al-Hourani H. Linkage between obesity leptin and breast cancer. *Breast Cancer (Auckl).* (2020) 14:1178223419898458. doi: 10.1177/1178223419898458

44. Andò S, Gelsomino L, Panza S, Giordano C, Bonofiglio D, Barone I, et al. Obesity, leptin and breast cancer: Epidemiological evidence and proposed mechanisms. *Cancers (Basel).* (2019) 11(1):62. doi: 10.3390/cancers11010062

45. Agurs-Collins T, Ross SA, Dunn BK. The many faces of obesity and its influence on breast cancer risk. *Front Oncol* (2019) 9:765. doi: 10.3389/fonc.2019.00765

46. Bulun SE, Chen D, Moy I, Brooks DC, Zhao H. Aromatase, breast cancer and obesity: a complex interaction. *Trends Endocrinol Metab* (2012) 23:83–9. doi: 10.1016/j.tem.2011.10.003

47. Feola A, Ricci S, Kouidhi S, Rizzo A, Penon A, Formisano P, et al. Multifaceted breast cancer: the molecular connection with obesity. *J Cell Physiol* (2017) 232(1):69–77. doi: 10.1002/jcp.25475

48. Gross AL, Newschaffer CJ, Hoffman-Bolton J, Rifai N, Visvanathan K. Adipocytokines, inflammation, and breast cancer risk in postmenopausal women: a prospective study. *Cancer Epidemiol Biomarkers Prev* (2013) 22:1319–24. doi: 10.1158/1055-9965.EPI-12-1444

49. Saxena NK, Sharma D. Multifaceted leptin network: the molecular connection between obesity and breast cancer. *J Mammary Gland Biol Neoplasia.* (2013) 18(3-4):309–20. doi: 10.1007/s10911-013-9308-2

50. Bhardwaj P, Brown KA. Obese adipose tissue as a driver of breast cancer growth and development: update and emerging evidence. *Front Oncol* (2021) 11:638918. doi: 10.3389/fonc.2021.638918

51. Carter JC, Church FC. Mature breast adipocytes promote breast cancer cell motility. *Exp Mol Pathol* (2012) 92(3):312–7. doi: 10.1016/j.yexmp.2012.03.005

52. Wu Q, Li B, Li Z, Li J, Sun S, Sun S. Cancer-associated adipocytes: key players in breast cancer progression. *J Hematol Oncol* (2019) 12(1):95. doi: 10.1186/s13045-019-0778-6

53. Bowers LW, Cavazos DA, Maximo IXF, Brenner AJ, Hursting SD, DeGraffenried LA. Obesity enhances nongenomic estrogen receptor crosstalk with the PI3K/Akt and MAPK pathways to promote *in vitro* measures of breast cancer progression. *Breast Cancer Res* (2013) 15(4):R59. doi: 10.1186/bcr3453

54. McCullough LE, Chen J, Cho YH, Khankari NK, Bradshaw PT, White AJ, et al. DNA Methylation modifies the association between obesity and survival after breast cancer diagnosis. *Breast Cancer Res Treat* (2016) 156(1):183–94. doi: 10.1007/s10549-016-3724-0

55. Martínez – Reza I, Díaz L, Barrera D, Segovia – Mendoza M, Pedraza – Sánchez S, Soca – Chafre G, et al. Calcitriol inhibits the proliferation of triple negative breast cancer cells through a mechanism involving the proinflammatory cytokines IL-1β and THF-α. *J Immunol Res* (2019) 2019:6384278. doi: 10.1155/2019/6384278

56. Thabet RH, Gomaa AA, Matalqah LM, Shalaby EM. Vitamin d: An essential adjuvant therapeutic agent in breast cancer. *J Int Med Res* (2022) 50 (7):30000605221113800. doi: 10.1177/03000605221113800

57. Malhotra GK, Zhao X, Band H, Band V. Histological, molecular and functional subtypes of breast cancers. *Cancer Biol Ther* (2010) 10:10, 955–960. doi: 10.4161/cbt.10.10.13879

58. Comşa Ş, Cîmpean AM, Raica M. The story of MCF-7 breast cancer cell line: 40 years of experience in research. *Anticancer Res* (2015) 35(6):3147–54.

59. Zhang X, Bolt M, Guertin MJ, Chen W, Zhang S, Cherrington BD, et al. Peptidylarginine deiminase 2-catalyzed histone H3 arginine 26 citrullination facilitates estrogen receptor α target gene activation. *Proc Natl Acad Sci U S A.* (2012) 109(33):13331–6. doi: 10.1073/pnas.1203280109

60. Ma F, Li W, Liu C, Li W, Yu H, Lei B, et al. MiR-23a promotes TGF-β1-Induced EMT and tumor metastasis in breast cancer cells by directly targeting CDH1 and activating wnt/β-catenin signaling. *Oncotarget* (2017) 8(41):69538. doi: 10.18632/oncotarget.18422

61. Johnson AR, Milner JJ, Makowski L. The inflammation highway: metabolism accelerates inflammatory traffic in obesity. *Immunol Rev* (2012) 249 (1):218–38. doi: 10.1111/j.1600-065X.2012.01151.x

62. Alam I, Tze Pin N, Larbi A. Does inflammation determine whether obesity is metabolically healthy or unhealthy? the aging perspective. *Mediators Inflamm* (2012) 456:1–14. doi: 10.1155/2012/456456

63. Tateya S, Kim F, Tamori Y. Recent advances in obesity-induced inflammation and insulin resistance. *Front Endocrinol (Lausanne)* (2013) 4:93–107. doi: 10.3389/fendo.2013.00093

64. Cailleau R, Young R, Olivé M, Reeves WJJr. Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst* (1974) 53(3):661–74. doi: 10.1093/jnci/53.3.661

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 22 December 2022
DOI 10.3389/fonc.2022.989761

OPEN ACCESS

EDITED BY
Ariella Hanker,
University of Texas Southwestern Medical Center, United States

REVIEWED BY
Alejandro Zentella-Dehesa,
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico
Mujeeb Zafar Banday,
Government Medical College (GMC), India

*CORRESPONDENCE
Silvia J. Serrano-Gómez
sserrano@cancer.gov.co

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 08 July 2022
ACCEPTED 06 December 2022
PUBLISHED 22 December 2022

CITATION
Rey-Vargas L, Bejarano-Rivera LM, Mejia-Henao JC, Sua LF, Bastidas-Andrade JF, Ossa CA, Gutiérrez-Castañeda LD, Fejerman L, Sanabria-Salas MC and Serrano-Gómez SJ (2022) Association of genetic ancestry with HER2, GRB7 AND estrogen receptor expression among Colombian women with breast cancer.
Front. Oncol. 12:989761.
doi: 10.3389/fonc.2022.989761

Association of genetic ancestry with HER2, GRB7 AND estrogen receptor expression among Colombian women with breast cancer

Laura Rey-Vargas[1,2], Lina María Bejarano-Rivera[1], Juan Carlos Mejia-Henao[3], Luz F. Sua[4], Jhon Faustino Bastidas-Andrade[5], Carlos Andrés Ossa[6], Luz Dary Gutiérrez-Castañeda[7], Laura Fejerman[8], María Carolina Sanabria-Salas[1] and Silvia J. Serrano-Gómez[1,9]*

[1]Cancer Biology Research Group, National Cancer Institute of Colombia, Bogotá, Colombia, [2]Doctoral Program in Biological Sciences, Pontificia Universidad Javeriana, Bogotá, Colombia, [3]Oncological Pathology Research Group, National Cancer Institute of Colombia, Bogotá, Colombia, [4]Department of Pathology and Laboratory Medicine, Fundación Valle del Lili, and Faculty of Health Sciences, Universidad ICESI, Cali, Colombia, [5]Oncology Unit, Fundación San Pedro Hospital, Pasto, Colombia, [6]Cancer Institute, Las Américas Clinic, Medellín, Colombia, [7]Research Institute, Group of Basic Sciences in Health (CBS), Fundación Universitaria de Ciencias de la Salud (FUCS), Bogotá, Colombia, [8]Department of Public Health Sciences and Comprehensive Cancer Center, University of California Davis, Davis, CA, United States, [9]Research support and follow-up group, National Cancer Institute of Colombia, Bogotá, Colombia

Background: Our previous study reported higher mRNA levels of the human epidermal growth factor receptor 2 (HER2)-amplicon genes *ERBB2* and *GRB7* in estrogen receptor (ER)-positive breast cancer patients with relatively high Indigenous American (IA) ancestry from Colombia. Even though the protein expression of HER2 and GRB7 is highly correlated, they may also express independently, an event that could change the patients' prognosis. In this study, we aimed to explore the differences in ER, HER2 and GRB7 protein expression according to genetic ancestry, to further assess the clinical implications of this association.

Methods: We estimated genetic ancestry from non-tumoral breast tissue DNA and assessed tumoral protein expression of ER, HER2, and GRB7 by immunohistochemistry in a cohort of Colombian patients from different health institutions. We used binomial and multinomial logistic regression models to test the association between genetic ancestry and protein expression. Kaplan-Meier and log-rank tests were used to evaluate the effect of HER2/GRB7 co-expression on patients' survival.

Results: Our results show that patients with higher IA ancestry have higher odds of having HER2+/GRB7- breast tumors, compared to the HER2-/GRB7- subtype, and this association seems to be stronger among ER-positive tumors (ER+/HER2+/GRB7-: OR=3.04, 95% CI, 1.47-6.37, p<0.05). However, in the multivariate model this association was attenuated (OR=1.80, 95% CI, 0.72-4.44, p=0.19). On the other hand, it was observed that having a higher European ancestry patients presented lower odds of ER+/HER2+/GRB7- breast tumors, this association remained significant in the multivariate model (OR=0.36, 95% CI, 0.13 - 0.93, p= 0.0395). The survival analysis according to HER2/GRB7 co-expression did not show statistically significant differences in the overall survival and recurrence-free survival.

Conclusions: Our results suggest that Colombian patients with higher IA ancestry and a lower European fraction have higher odds of ER+/HER2+/GRB7- tumors compared to ER+/HER2-/GRB7- disease. However, this association does not seem to be associated with patients' overall or recurrence-free survival.

KEYWORDS

breast neoplasms, American native continental ancestry, receptor ErbB-2, GRB7 adaptor protein, estrogen receptor

Introduction

Breast cancer is the malignancy with the highest incidence (47.8 per 100,000) and mortality rates among women worldwide (13.6 per 100,000) (1). At the molecular level, it is a heterogeneous disease that has been classified into four major intrinsic subtypes (luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, and basal-like or triple negative) based on tumor's gene expression profiles (2). Each of these subtypes has a different clinical prognosis. Breast cancer luminal subtypes, characterized by the positive expression of estrogen receptor (ER) and progesterone receptor (PR), often show a well to moderately differentiated phenotype, a low cellular proliferation index, and are associated with a relatively good survival probability. On the other hand, basal-like and HER2-enriched subtypes, both negative for hormone receptors expression, often present an aggressive phenotype; these patients are frequently diagnosed at higher clinical stages with nodal involvement and larger tumors (3, 4).

Differences in the prevalence of breast tumor subtypes by population group have been widely described (5–7). Epidemiologic studies have consistently reported that non-Hispanic White (NHW) women have a higher prevalence of luminal A disease, whilst African American/Black (AA/B) and Hispanic/Latina women have a higher prevalence of basal-like and HER2-enriched subtypes (7–9). Possible contributors to these disparities include differences in the presentation of several reproductive (parity, duration of lactation and age at first birth) and socioeconomic risk factors (socioeconomic status and health insurance) among populations (10–15). Genetic ancestry proportion has also been linked to differences in clinical-pathological characteristics of breast cancer among these population groups, including differences in the distribution of breast cancer subtypes (16–19). A recent study conducted in Peruvian women, a population with relatively high average of Indigenous American (IA) ancestry proportion, reported a 20% increase in the odds of developing HER2-positive breast tumors per every 10% increment in the IA ancestry fraction (OR=1.20, 95% CI, 1.07-1.35, p=0.001) (20), and this association was especially strong for ER-negative tumors. We have also shown that Colombian women with higher IA ancestry (>36%) expressed higher mRNA levels of the *ERBB2* gene, although in this study, only ER-positive tumors were analyzed (21).

ERBB2 is located in the 17q12 region, at the so-called HER2 amplicon, and it is usually co-amplified with other genes such as *GRB7* (22). Additionally, HER2/GRB7 co-expression has been associated with resistance to anti-HER2 treatments and with poor prognosis (23–25). It has also been reported that HER2 may express independently from GRB7, and this event might confer a different prognosis (22). It is still unclear whether IA ancestry is associated with the co-expression of both proteins, and what it is the linkage between this association with ER expression. Additionally, the clinical implications of HER2/GRB7 co-expression in the Colombian population have not yet been explored. Therefore, in this study we tested differences in ER, HER2 and GRB7 protein expression according to genetic

ancestry, as well as the association of different combinations of expression of these proteins with breast cancer survival in Colombian women.

Materials and methods

Sample selection

We revised the clinical-pathological data from breast cancer patients diagnosed between 2013 and 2015 at the Colombian National Cancer Institute (NCI) in Bogotá D.C, a national reference center for cancer treatment that admits patients from all country regions. A cohort of 361 patients were selected according to the following inclusion criteria: 1) histologically confirmed diagnosis of invasive ductal carcinoma (IDC), 2) availability of formalin-fixed paraffin-embedded (FFPE) tissue blocks that contained at least 10% of tumor content from mastectomies or breast-conserving surgeries, and 3) availability of FFPE blocks with no-tumor content. Patients with *in situ* carcinoma were excluded. In order to enrich the population sample with patients from different regions and different genetic ancestry proportions, we invited other institutions to participate in the study. Breast cancer patients diagnosed at the San Pedro Hospital (SPH) (n=55), the Fundación Valle de Lili (FVL) University Hospital (n=73), and Las Américas Clinic (LAC) (n=28), were also included under the same selection criteria. Biospecimens from a total of 517 patients were included in this study. This research was approved by the ethics committee from all four institutions, and according to the Colombian laws, it was considered that no informed consent was required.

Immunohistochemistry

Immunohistochemistry (IHC) assays were performed on 3 μm thick sections from a single FFPE surgery block with the highest tumor content, using monoclonal antibodies for ER (clone SP1 Roche 05278406001), PR (clone 1E2 Roche 05278392001), HER2 (clone 4B5 Roche 05278368001), Ki67 (clone 30-9 Roche 05278384001) and GRB7 (A-12 sc-376069, Santa Cruz Biotechnology), using the Roche Benchmark XT automated slide preparation system (Roche Ltd., Switzerland). Positive controls were included and 3,3′ diaminobenzidine (DAB) was used as the chromogen.

A single pathologist analyzed the IHC expression of the ER, PR, HER2 and Ki67 biomarkers. Status of hormone receptors was considered positive when they exceeded 1% of nuclear staining in tumor cells. HER2 evaluation followed the recommendations of the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guideline (26) and was defined as: positive (3+) for complete and intense circumferential membrane within >10% of tumor cells; ambiguous (2+) for incomplete and/or weak/moderate circumferential membrane staining within > 10% of tumor cells, or complete membrane staining but within ≤10% of tumor cells; negative (1+) for incomplete faint membrane staining within >10% of tumor cells; and negative (0+) for absence of staining. HER2 ambiguous (2+) cases with no confirmatory fluorescence *in situ* hybridization (FISH)/ chromogenic *in situ* hybridization (CISH) test were excluded from the analysis. GRB7 expression was assessed as the percentage of tumor cells with positive membrane/cytoplasmic staining. Cases with ≥10% of GRB7 membrane/cytoplasmic staining were defined as positive, while the remaining cases (<10% staining) were defined as negative.

Genetic ancestry estimation

DNA was extracted from non-tumor paraffin blocks using the AllPrep DNA/RNA FFPE kit (Qiagen, Inc., Valencia, CA, USA) and the RecoverAll Total Nucleic Acid for FFPE kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Nucleic acid concentration was quantified by NanoDrop ND1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). A panel of 106 Single Nucleotide Polymorphisms (SNPs) previously validated as Ancestry Informative Markers (AIMs) (21) were genotyped at the University of Minnesota Genomics Center, using the Sequenom technology. SNPs with a call rate <90% or that deviated from Hardy-Weinberg equilibrium were removed from the analysis, leaving 87 SNPs for individual genetic ancestry estimation. A total of 495 samples were genotyped and 381 remained after excluding samples with a genotype call rate <85% (NCI= 308/361; FVL= 29/73; SPH= 44/55; LAC= 0/28).

We genotyped 10 duplicate pairs and the overall discordance rate was 0. Quality control of the genotyped data was performed in PLINK 1.9 (27), and the software Admixture 1.3 (28) was used under an admixture model (k=3) to estimate IA, European and African ancestry proportions.

Statistical analysis

All statistical analyses were performed using the RStudio software version 1.2.5019. Continuous variables presented a non-normal distribution and were reported as medians and interquartile ranges (IQR). Categorical variables were summarized as absolute and relative frequencies. We applied a

Kruskal–Wallis test to assess differences in genetic ancestry fractions according to the status of ER, HER2 and GRB7, and tumor subtype (ER/HER2); and a Chi-square (X^2) test to assess differences in categorical variables. Unknown and not classifiable categories were not included in statistical analysis.

We used a univariate logistic regression model to evaluate the association between the expression of ER, HER2 and GRB7 per every 25% increase in genetic ancestry fractions. A univariate multinomial logistic regression model was used to assess the association between genetic ancestry and: (1) Breast cancer subtypes categorized by ER/HER2 expression and (2) ER/HER2/GRB7 co-expression status. For the multivariate logistic regression model, we included potential cofounding variables such as health institution, clinical stage, and age at diagnosis.

We evaluated differences in overall survival (OS) and disease-free survival (DFS) according to HER2/GRB7 co-expression status using the Kaplan-Meier and log rank test. OS was calculated from the date of diagnosis to the date of death or last follow-up. DFS was calculated from the date of surgery to the date of the first recurrence (local, regional, or distant relapse) or last follow-up. Differences were considered statistically significant if $p<0.05$.

Results

Patients' characteristics

Patients' clinical-pathological characteristics according to health institution are described in Table 1. We observed statistically significant differences in all variables evaluated. At the NCI, we observed a higher percentage of patients diagnosed with breast cancer over the age of 50 (77.0%) with late clinical stages (III/IV: 45.7%) and positive histological invasion (51.5%). Most of the patients from the FVL presented poorly differentiated tumors (Scarff-Bloom Richardson III: 49.2%), had lymph node involvement (76.9%), and 42.5% had deceased at the time of the study. A higher percentage of patients from the SPH presented moderately differentiated tumors (Scarff-Bloom Richardson II: 50.9%) and had the highest proportion of recurrence (32.7%) among all health institutions. Overall, patients from the LAC presented favorable clinical-pathological features such as early clinical stages (I: 60.7%), negative histological invasion (75.0%), and no clinical recurrence at the time of the study.

Statistically significant differences between health institutions were observed for ER/HER2 expression ($p<0.001$) and Ki67 status ($p<0.001$). The ER+/HER2- subtype was the most prevalent in all patients, although it was especially frequent among patients from the LAC (82.1%). SPH had a higher number of patients with HER2+ tumors (ER+/HER2+: 20%, ER-/HER2+: 16.4%) and ER-/HER2- tumors were more frequently observed at the NCI (15.2%) compared to other health institutions.

Genetic ancestry distribution according to ER, HER2 and GRB7 breast tumor expression

Genetic ancestry data was available for 73.7% of the cases (381/517). The average genetic ancestry proportions for the European, IA, and African components were 48.9%, 42.1%, and 8.9%, respectively (Figure 1). We observed statistically significant differences in genetic ancestry fractions by health institution (Table 1). European and IA ancestry components were significantly higher in the NCI (0.51) and the SPH (0.58), respectively, whereas African ancestry was higher in the FVL (0.12).

We analyzed differences in genetic ancestry by ER, HER2 and GRB7 status (Table 2). We observed that both HER2 and GRB7 positive cases presented a higher IA ancestry, compared to the negative group (HER2 positive (3+): 0.44 vs. negative (0+/1+): 0.40, $p=0.003$; GRB7-positive: 0.43 vs negative: 0.41, $p=0.019$). HER2-negative cases also showed a significantly higher median of European ancestry, compared to the HER2-positive group (0.49 vs 0.44, respectively, $p=0.024$). Regarding the African component, no statistically significant differences were observed in HER2 and GRB7 expression by this fraction. Similarly, ER expression also did not show any statistically significant changes by genetic ancestry.

The co-expression analysis of ER/HER2 showed a higher median of IA ancestry in ER+/HER2+ tumors compared to ER+/HER2- group (0.45 vs. 0.40, $p=0.023$). Interestingly, when GRB7 was included in the co-expression analysis, we observed the highest IA ancestry fraction in the ER+/HER2+/GRB7- group (0.55 vs. 0.40, $p=0.02$), and the lowest European ancestry values (0.35 vs. 0.49, $p=0.009$), compared to the ER+/HER2-/GRB7- group. The co-expression analysis (ER/HER2/GRB7) did not show any statistically significant differences by African ancestry (Table 2).

Association of genetic ancestry with ER/HER2/GRB7 breast tumor expression

We assessed the association between genetic ancestry and the expression of ER, HER2, GRB7, and their co-expression in a multivariable model. This analysis showed that every 25% increase in the IA ancestry fraction led to a 1.89 increase in the odds of having HER2-positive breast tumors (OR=1.89, 95% CI, 1.22–2.94, $p=0.0043$); on the contrary, higher European ancestry was found associated with lower odds of HER2-positive breast tumors (OR=0.58, 95% CI, 0.36 – 0.91, $p=0.0204$). After adjusting for potential covariates, the association between IA and European ancestry with HER2 expression was no longer statistically significant (Table 3 and Supplementary Table S1). On the other hand, no statistically significant associations were found between African ancestry and the expression of these proteins (Supplementary Table S2).

TABLE 1 Clinical-pathological characteristics of patients by health institution.

	NCI (n = 361)	FVL (n = 73)	SPH (n = 55)	LAC (n = 28)	*p* value
	N (%)	N (%)	N (%)	N (%)	
Age of diagnosis					
≤50 years	83 (23.0)	27 (37.0)	25 (45.5)	6 (21.4)	<0.001[a]
>50 years	278 (77.0)	40 (54.8)	30 (54.5)	21 (75.0)	
Unknown	0 (0.0)	6 (8.2)	0 (0.0)	1 (3.6)	
AJCC Clinical stage					
I (I, Ia, Ib)	40 (11.1)	10 (13.7)	4 (7.3)	17 (60.7)	<0.001[a]
II (IIa, IIb)	156 (43.2)	29 (39.7)	30 (54.5)	10 (35.7)	
III (IIIa, IIIb, IIIc)/IV	165 (45.7)	28 (38.4)	18 (32.7)	1 (3.6)	
Unknown	0 (0.0)	6 (8.2)	3 (5.5)	0 (0.0)	
Scarff-Bloom Richardson					
I	30 (8.3)	9 (13.8)	21 (38.2)	8 (28.6)	<0.001[a]
II	192 (53.2)	24 (36.9)	28 (50.9)	19 (67.9)	
III	138 (38.2)	32 (49.2)	4 (7.3)	1 (3.6)	
Unknown	1 (0.3)	0 (0.0)	2 (3.6)	0 (0.0)	
Tumor size					
≤ 20 mm	96 (26.6)	26 (35.6)	18 (32.7)	0 (0.0)	0.206[a]
> 20 mm	253 (70.1)	46 (63.0)	28 (50.9)	1 (3.6)	
Unknown	12 (3.3)	1 (1.4)	9 (16.4)	27 (96.4)	
Histological invasion					
Negative	139 (38.5)	28 (38.4)	26 (47.3)	21 (75.0)	0.005[a]
Positive	186 (51.5)	45 (61.6)	25 (45.5)	7 (25.0)	
Unknown	36 (10.0)	0 (0.0)	4 (7.3)	0 (0.0)	
Lymph node involvement					
Negative	188 (52.1)	12 (23.1)	11 (25.0)	1 (10.0)	<0.001[a]
Positive	173 (47.9)	40 (76.9)	33 (75.0)	9 (90.0)	
Unknown	0 (0.0)	21 (28.8)	11 (20.0)	18 (64.3)	
Ki67 status					
High (≥20%)	213 (59.0)	29 (39.7)	31 (56.4)	4 (14.3)	<0.001[a]
Low (<20%)	148 (41.0)	44 (60.3)	23 (41.8)	24 (85.7)	
Unknown	0 (0.0)	0 (0.0)	1 (1.8)	0 (0.0)	
ER/HER2 tumor subtype					
ER+/HER2-	204 (56.5)	50 (68.5)	26 (47.3)	23 (82.1)	<0.001[a]
ER+/HER2+	34 (9.4)	8 (11.0)	11 (20.0)	3 (10.7)	
ER-/HER2+	21 (5.8)	7 (9.6)	9 (16.4)	1 (3.6)	
ER-/HER2-	55 (15.2)	8 (11.0)	5 (9.1)	1 (3.6)	

(Continued)

10.3389/fonc.2022.989761

TABLE 1 Continued

	NCI (n = 361)	FVL (n = 73)	SPH (n = 55)	LAC (n = 28)	*p* value
	N (%)	N (%)	N (%)	N (%)	
Not classifiable	47 (13.0)	0 (0.0)	4 (7.3)	0 (0.0)	
Clinical recurrence					
Negative	247 (68.4)	24 (32.9)	30 (54.5)	28 (100.0)	<0.001[a]
Positive	87 (24.1)	22 (30.1)	18 (32.7)	0 (0.0)	
Unknown	27 (7.5)	27 (37.0)	7 (12.7)	0 (0.0)	
Vital state					
Alive	277 (76.7)	41 (56.2)	32 (58.2)	28 (100.0)	<0.001[a]
Deceased	83 (23.0)	31 (42.5)	6 (10.9)	0 (0.0)	
Unknown	1 (0.3)	1 (1.4)	17 (30.9)	0 (0.0)	
Genetic ancestry (median [IQR])					
European ancestry fraction	0.51 [0.43, 0.59]	0.48 [0.33, 0.62]	0.36 [0.26, 0.44]	Not available	<0.001[b]
IA ancestry fraction	0.40 [0.32, 0.48]	0.32 [0.19, 0.42]	0.58 [0.51, 0.69]	Not available	<0.001[b]
African ancestry fraction	0.07 [0.03, 0.12]	0.12 [0.03, 0.21]	0.03 [0.00, 0.08]	Not available	<0.001[b]

NCI, National Cancer Institute; FVL, Fundación Valle de Lili; SPH, San Pedro Hospital; LAC, Las Américas Clinic; ER, estrogen receptor; IA, Indigenous American; IQR, interquartile range.
Statistical tests: [a]Fisher's exact test, [b]Kruskal-Wallis
Unknown and not classifiable categories were not included in the statistical analysis.

Furthermore, we observed that higher levels of IA ancestry and a lower European ancestry fraction was associated with higher odds of ER+/HER2+ breast tumors (IA ancestry fraction: OR= 1.93, 95% CI, 1.13-3.32, p=0.0154; European ancestry fraction: OR= 0.46, 95% CI, 0.26-0.82, p= 0.00882). When GRB7 was included in the co-expression analysis (ER/HER2/GRB7), a stronger association was observed between both IA and European ancestry fractions with ER+/HER2+/GRB7- tumors (IA ancestry fraction: OR= 3.04, 95% CI, 1.47 – 6.37, p=0.00245; European ancestry fraction: OR= 0.23, 95% CI, 0.1-0.52, p=0.000448) (Table 3 and Supplementary Table S1). However, in the multivariate model the reported association between genetic ancestry and the ER+/HER2+/GRB7- subtype remained statistically significant only for the European component (OR= 0.36, 95% CI, 0.13-0.93, p= 0.0395) (Supplementary Table S1). Regarding the African component, the co-expression analysis for ER+/HER2+/GRB7- did not show any statistically significant associations with this ancestry fraction (Supplementary Table S2).

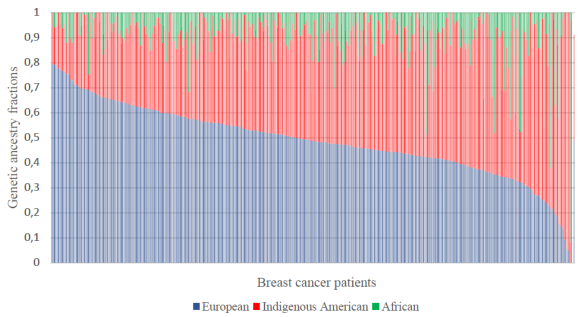


FIGURE 1
Genetic ancestry distribution for 381 breast cancer patients from Columbia. Each patient is represented by a vertical bar at the x-axis.

Differences in clinical–pathological characteristics and outcomes by HER2/GRB7 co-expression

We explored differences in the presentation of clinical-pathological features among breast cancer patients according to HER2/GRB7 status (Supplementary Table S3). This analysis showed that HER2+/GRB7- and HER2+/GRB7+ patients were more frequently diagnosed under the age of 50 (39.5% and 39.3%

10.3389/fonc.2022.989761

TABLE 2 Differences of genetic ancestry fractions according to the status of ER, HER2 and GRB7, and their co-expression.

	N=381	European (median [IQR])	*p* value	IA (median [IQR])	*p* value	African (median [IQR])	*p* value
ER status							
Positive	293	0.48 [0.42, 0.59]	0.651	0.41 [0.32, 0.51]	0.396	0.07 [0.03, 0.12]	0.536
Negative	88	0.50 [0.41, 0.57]		0.42 [0.34, 0.51]		0.06 [0.02, 0.11]	
HER2 status*							
Positive (3+)	66	0.44 [0.34, 0.57]	0.024	0.44 [0.37, 0.57]	0.003	0.06 [0.03, 0.13]	0.362
Negative (0+/1+)	273	0.49 [0.42, 0.58]		0.40 [0.32, 0.50]		0.07 [0.03, 0.12]	
GRB7 status							
Positive	52	0.47 [0.42, 0.56]	0.256	0.43 [0.40, 0.54]	0.019	0.06 [0.02, 0.10]	0.276
Negative	329	0.49 [0.42, 0.59]		0.41 [0.32, 0.50]		0.07 [0.03, 0.12]	
ER/HER2 subtype*							
ER+/HER2-	218	0.49 [0.42, 0.59]	0.053	0.40 [0.31, 0.50]	0.023	0.07 [0.03, 0.12]	0.452
ER+/HER2+	39	0.43 [0.34, 0.53]		0.45 [0.40, 0.55]		0.06 [0.03, 0.15]	
ER-/HER2+	27	0.50 [0.38, 0.60]		0.43 [0.34, 0.57]		0.05 [0.01, 0.09]	
ER-/HER2-	55	0.51 [0.41, 0.56]		0.42 [0.33, 0.50]		0.06 [0.03, 0.12]	
ER/HER2/GRB7 co-expression⁺							
ER+/HER2-/GRB7-	217	0.49 [0.43, 0.59]	0.009	0.40 [0.31, 0.49]	0.020	0.07 [0.03, 0.12]	0.558
ER+/HER2+/GRB7-	15	0.35 [0.27, 0.42]		0.55 [0.42, 0.63]		0.06 [0.03, 0.13]	
ER+/HER2+/GRB7+	24	0.47 [0.42, 0.56]		0.43 [0.39, 0.51]		0.07 [0.03, 0.15]	
ER-/HER2+/GRB7+	20	0.45 [0.36, 0.57]		0.48 [0.40, 0.58]		0.05 [0.01, 0.07]	
ER-/HER2+/GRB7-	7	0.60 [0.47, 0.62]		0.35 [0.28, 0.47]		0.07 [0.04, 0.11]	
ER-/HER2-/GRB7-	54	0.51 [0.41, 0.56]		0.42 [0.33, 0.50]		0.07 [0.03, 0.12]	

IA, Indigenous American; IQR, interquartile range.
*HER2 equivocal (2+) cases with no confirmatory result (n=42) were excluded from the analysis.
⁺HER2-/GRB7+ tumors (n=2) were excluded from the analysis because of low representation.
Statistical test: Kruskal-Wallis.

TABLE 3 Association per every 25% increase in IA ancestry with ER/HER2/GRB7 expression.

	Univariate		Multivariate	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
ER status				
Negative	1.00		1.00	
Positive	0.87 (0.58-1.39)	0.5065	1.02 (0.63 - 1.66)	0.9180
HER2 status*				
Negative (0+/1+)	1.00		1.00	
Positive (3+)	1.89 (1.22 - 2.94)	0.0043	1.48 (0.89 - 2.46)	0.1289
GRB7 status				
Negative	1.00		1.00	
Positive	1.59 (0.98 - 2.55)	0.0566	1.39 (0.78 - 2.44)	0.2559
ER/HER2 subtype				
ER+/HER2-	1.00		1.00	
ER+/HER2+	1.93 (1.13 - 3.32)	0.0154	1.61 (0.84 - 3.09)	0.1480
ER-/HER2+	1.72 (0.92 - 3.16)	0.0795	1.18 (0.50 - 2.67)	0.6808
ER-/HER2-	1.04 (0.63 - 1.71)	0.8528	1.07 (0.58 - 1.92)	0.8144
ER/HER2/GRB7 co-expression^{+}				
ER+/HER2-/GRB7-	1.00		1.00	
ER+/HER2+/GRB7-	3.04 (1.47 - 6.37)	0.00245	1.80 (0.72 - 4.44)	0.1950
ER+/HER2+/GRB7+	1.31 (0.64 - 2.58)	0.436	1.36 (0.56 - 3.20)	0.477
ER-/HER2+/GRB7+	2.14 (1.07 - 4.22)	0.0279	1.43 (0.52 - 3.78)	0.473
ER-/HER2+/GRB7-	0.88 (0.21 - 2.84)	0.8510	0.82 (0.13 - 3.63)	0.809
ER-/HER2-/GRB7-	1.05 (0.63 - 1.73)	0.8294	1.06 (0.58 - 1.91)	0.8317

OR, odd ratio; CI, confidence interval; IA, Indigenous American.
*HER2 equivocal (2+) cases with no confirmatory result (n=42) were excluded from the analysis.
$^{+}$HER2-/GRB7+ tumors (n=2) were excluded from the analysis because of low representation.
The multivariate model was adjusted by health institution, age of diagnosis and clinical stage.

vs. 25.7%, respectively, p=0.036) and presented higher proliferation rates (Ki67 status ≥20%: 71.1% and 87.5% vs. 45.5%, respectively, p<0.001), compared to HER2-/GRB7- patients. However, we observed a higher frequency of less differentiated tumors only for patients with the HER2/GRB7 co-expression, compared to both HER2+/GRB7- and HER2-/GRB7- patients (Scarff-Bloom Richardson III: 53.6% vs. 36.8% and 30.5%, respectively, p=0.007). No statistically significant differences were found for OS and RFS by the co-expression status (Supplementary Figures S1).

Discussion

We aimed to assess the association between genetic ancestry and the protein expression of ER, HER2, and GRB7, along with its clinical implications in Colombian patients with breast cancer. In an effort to have a better representation of the three main ancestry fractions in Colombia (European, IA, and African), breast cancer patients from four different health institutions around the country were included. As a result, we observed a great heterogeneity in the patient's clinicopathological features according to health institution. This is a problem hospital-based studies in Latin-America often face as a consequence of the different treatment protocols used for cancer management in each health institution, but also the profile of people that live in each specific area. We observed a higher proportion of patients diagnosed at earlier stages in some institutions, while in others, patients presented worse disease outcomes, seen as higher recurrences. This might be closely related to patient's socioeconomic factors who attend each institution, like

insurance regime (subsidized and contributory) and access to health services (29–31). All of these are challenges that need to be addressed in further studies in Latin-American countries (32).

Differences in genetic ancestry were also observed according to health institution. Patients that came from health centers in the Andean region, such as the NCI, showed the highest European ancestry, whereas patients from the FVL and SPH had higher proportions of African and IA ancestry components respectively. This is expected as the FVL is located in Cali, a city in the Colombian Pacific region, where a strong influx of African population happened during colonization (33, 34), explaining the highest fraction of African ancestry in patients from this health institution. On the other hand, the SPH, located in the city of Pasto, has a strong IA influence, and receives more patients from rural areas, explaining the high fraction of IA ancestry in this region (33).

As previously reported, our findings suggest that breast cancer patients with a higher IA ancestry and a lower European component might have an increased risk of developing HER2-positive tumors. As for GRB7, a gene commonly co-expressed with HER2 due to their proximity on chromosome 17, position q12, we only found it associated with IA and European ancestry when we analyzed its expression alone. However, when we analyzed it in a HER2/GRB7 co-expression model, the association was observed only for HER2+/GRB7- tumors, suggesting that the association found with genetic ancestry is mostly driven by the HER2 status. As for the hormone receptor, no statistically significant associations were observed between ER expression alone and genetic ancestry. However, when we analyzed it in a model together with HER2 and GRB7 co-expression, we observed a stronger association with IA and European ancestry among ER-positive tumors. Based on these results, we hypothesize that this protein might have a potential role in the association between genetic ancestry and the protein expression of HER2. Further studies are needed to keep exploring these results.

Population-based studies have showed that Hispanic/Latina women from United States with breast cancer, have a higher proportion of HER2-positive tumors when compared to NHW patients (7, 35, 36). Results from two hospital-based studies from Latin America have consistently reported an association between IA ancestry fraction and HER2 expression (20, 21). The first one, conducted in Colombian breast cancer patients with luminal subtypes reported a higher expression of the *ERBB2* gene in patients with higher IA ancestry (>36%) (21), while the second one, conducted in a large population of Peruvian women, consistently reported a higher risk of developing HER2-positive tumors per every 10% increment in the IA ancestry (20). Although we did not find the association with the IA ancestry component in the multivariate model, we still observed a consistent trend. On the other hand, this association was still significant in the multivariate model for the European component, which might be due to the higher contribution of this particular ancestry component among the Colombian population (16, 33). A positive correlation between ER-negative tumors and a higher African ancestry has been widely explored and documented before (19, 37), however, in this study we did not replicate these previous findings, possibly due to the lower representation of this specific component among our Colombian sample population.

So far, the genetic basis of this association remains unclear, however, as a possible explanatory mechanism, it has been proposed that there might be genetic variants located within ancestry-specific genomic regions, with the ability to regulate gene expression. These genetic variants are known as expression Quantitative Trait Loci (eQTLs) (38, 39). This hypothesis is supported by a large study across 33 cancer types from the Cancer Genome Atlas Project (TCGA), where they analyzed germline variant data of 9,899 cases, and reported the presence of ancestry-specific predisposition variants that were associated with an altered expression of the affected genes (e.g., *BRCA2* in samples of African ancestry) (40). Moreover, a genome-wide association study conducted in Latinas did find a genetic variant (rs140068132) that is mostly present in populations with IA ancestry, and is associated with a lower risk of ER-negative breast tumors (41, 42). Overall, these latter studies support the hypothesis that genetic ancestry can impact breast cancer phenotype. Further studies are still needed to explore the molecular mechanisms behind this association and to identify potential population-specific eQTLs that predispose Latina women to develop HER2-positive breast tumors.

Ours is the first study in Latin-American women with breast cancer to assess clinical prognosis according to the co-expression of these proteins. ER and HER2 are well-known biomarkers in breast cancer, widely used for prognosis assessment and to guide treatment protocols (43, 44). GRB7, on the other side, has been less explored in the context of breast cancer. Studies that have assessed its expression in tumor samples, suggest the role of GRB7 as an adaptor protein that binds to tyrosine kinase receptors like HER2, to amplify its signal and mediate the activation of several downstream proteins involved in cell migration and survival pathways (45). It has been shown that tumors with HER2/GRB7 co-expression present even higher nuclear grades and lower survival rates, compared with tumors that only express HER2 (46, 47). However, we did not find any significant differences in the OS and DFS according to the co-expression status, suggesting that GRB7 might not impact the prognosis of breast cancer patients. The introduction of anti-HER2 therapies such as trastuzumab and pertuzumab to treatment schemes for HER2-positive breast tumors have significantly improved the patients' prognosis (48). This could also be related with the fact that no statistically significant differences in the patients' survival were found by HER2/GRB7 co-expression status.

During this study, we encountered several limitations. The first and most important one was the heterogeneity of the

population included by health institution in terms of clinical-pathological characteristics but also by genetic ancestry. As has been reported before, genetic ancestry not only reflects the genetic profile derived from our ancestors, but is also an indirect reflector of other non-genetic exposures related to lifestyle and environmental risk factors associated with human behavior around populations (18, 49–51). It is possible that such factors can impact the tumor phenotype and the course of the disease, acting as cofounding variables. Another limitation was the small sample size, especially when we evaluated ER/HER2/GRB7 co-expression groups, which may have reduced the study's statistical power, and limited the opportunity to find biological associations. Additionally, we had a low representation of HER2-positive tumors in our sample. We highlight the need for population-based cancer registries in Latin-America to avoid the effects of unmeasured environmental exposure factors on research studies conducted in undeveloped countries (52).

In conclusion, our results suggest that Colombian patients with higher IA ancestry and a lower European component might have higher odds of developing breast luminal tumors with HER2 overexpression but no GRB7, compared to other subtypes. However, this association does not seem to have an impact on the patients' overall or recurrence-free survival. We highlight the need to conduct new investigations from population-based cancer studies in Latin America to confirm this association and to explore the genetic basis of these findings. This is an important matter as nowadays the healthcare system is moving towards a more personalized medicine model, and it is crucial to keep gathering as much scientific evidence as possible of the potential effects of genetic ancestry on breast cancer phenotype so that, in the future, this variable may contribute to the decision-making process on patients' disease management.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the National Cancer Institute of Colombia. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements

Author contributions

The concept of the study was conceived by SS-G and MS-S. LR-V, LB-R, JM-H, LS, JB-A, CO, and LG-C contributed to data collection. Data analysis and interpretation were performed by LR-V, LB-R, LF, MS-S and SS-G. LR-V wrote the manuscript in collaboration with SS-G. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Colombian National Cancer Institute (C19010300-411 and C19010300-476) and the Science and Technology Colombian Ministry – MINCIENCIAS (Programa de Becas de Excelencia Doctoral del Bicentenario 2do corte).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.989761/full#supplementary-material

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2021) 71(3):209–49. doi: 10.3322/caac.21660

2. Turashvili G, Brogi E. Tumor heterogeneity in breast cancer. *Front Med Front Med S.A.;* (2017) 4:227. doi: 10.3389/fmed.2017.00227

3. Prat A, Carey LA, Adamo B, Vidal M, Tabernero J, Cortés J, et al. Molecular features and survival outcomes of the intrinsic subtypes within HER2-positive breast cancer. *J Natl Cancer Inst* (2014) 106(8):dju152. doi: 10.1093/jnci/dju152

4. Prat A, Pineda E, Adamo B, Galván P, Fernández A, Gaba L, et al. Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast.* (2015) 24: S26–35. doi: 10.1016/j.breast.2015.07.008

5. Sweeney C, Bernard PS, Factor RE, Kwan ML, Habel LA, Quesenberry CP, et al. Intrinsic subtypes from PAM50 gene expression assay in a population-based breast cancer cohort: Differences by age, race, and tumor characteristics. *Cancer Epidemiol Biomarkers Prev* (2014) 23(5):714–24. doi: 10.1158/1055-9965.EPI-13-1023

6. Serrano-Gómez SJ, Fejerman L, Zabaleta J. Breast cancer in latinas: A focus on intrinsic subtypes distribution. *Cancer Epidemiol Biomarkers Prev* (2018) 27 (1):3–10. doi: 10.1158/1055-9965.EPI-17-0420

7. Howlader N, Altekruse SF, Li CI, Chen VW, Clarke CA, Ries LAG, et al. US Incidence of breast cancer subtypes defined by joint hormone receptor and Her2 status. *J Natl Cancer Inst* (2014) 106(5):dju055. doi: 10.1093/jnci/dju055

8. Hines LM, Risendal B, Byers T, Mengshol S, Lowery J, Singh M. Ethnic disparities in breast tumor phenotypic subtypes in Hispanic and non-Hispanic white women. *J Women's Heal* (2011) 20(10):1543–50. doi: 10.1089/jwh.2010.2558

9. Rey-Vargas L, Sanabria-Salas MC, Fejerman L, Serrano-Gómez SJ. Risk factors for triple-negative breast cancer among latina women. *Cancer Epidemiol Biomarkers Prev* (2019) 28(11):1771–83. doi: 10.1158/1055-9965.EPI-19-0035

10. Martínez ME, Gomez SL, Tao L, Cress R, Rodriguez D, Unkart J, et al. Contribution of clinical and socioeconomic factors to differences in breast cancer subtype and mortality between Hispanic and non-Hispanic white women. *Breast Cancer Res Treat* (2017) 166(1):185–93. doi: 10.1007/s10549-017-4389-z

11. Parise CA, Caggiano V. Disparities in race/ethnicity and socioeconomic status: risk of mortality of breast cancer patients in the California cancer registry, 2000-2010. *BMC Cancer.* (2013) 13:1. doi: 10.1186/1471-2407-13-449

12. Sweeney C, Baumgartner KB, Byers T, Giuliano AR, Herrick JS, Murtaugh MA, et al. Reproductive history in relation to breast cancer risk among Hispanic and non-Hispanic white women. *Cancer Causes Control.* (2008) 19(4):391–401. doi: 10.1007/s10552-007-9098-1

13. Gilliland FD, Hunt WC, Baumgartner KB, Crumley D, Nicholson CS, Fetherolf J, et al. Reproductive risk factors for breast cancer in hispanic and non-hispanic white women: The new mexico women's health study. *Am J Epidemiol.* (1998) 148(7):683–92. doi: 10.1093/aje/148.7.683

14. Chen L, Li CI, Tang MC, Porter P, Hill DA, Charles L, et al. Reproductive factors and risk of luminal, HER2-overexpressing and triple negative breast cancer among multiethnic women. *Cancer Epidemiol Biomarkers Prev* (2017) 25(9):1297–304. doi: 10.1158/1055-9965.EPI-15-1104

15. Hines LM, Risendal B, Slattery ML, Baumgartner KB, Giuliano AR, Byers T. Differences in estrogen receptor subtype according to family history of breast cancer among Hispanic, but not non-Hispanic white women. *Cancer Epidemiol Biomarkers Prev* (2008) 30(2):83–8. doi: 10.1158/1055-9965.EPI-08-0504

16. Serrano-Gomez SJ, Sanabria-Salas MC, Hernández-Suarez G, García O, Silva C, Romero A, et al. High prevalence of luminal b breast cancer intrinsic subtype in Colombian women. *Carcinogenesis* (2016) 37(7):669–76. doi: 10.1093/carcin/bgw043

17. Hines LM, Sedjo RL, Byers T, John EM, Fejerman L, Stern MC, et al. The interaction between genetic ancestry and breast cancer risk factors among Hispanic women: The breast cancer health disparities study. *Cancer Epidemiol Biomarkers Prev* (2017) 26(5):692-701. doi: 10.1158/1055-9965.EPI-16-0721

18. Fejerman L, John EM, Huntsman S, Beckman K, Perez-stable E, Burchard EG, et al. Genetic ancestry and risk of breast cancer among US latinas. *Cancer Res* (2009) 68(23):9723–8. doi: 10.1158/0008-5472.CAN-08-2039

19. Huo D, Hu H, Rhie SK, Gamazon ER, Cherniack AD, Liu J, et al. Comparison of breast cancer molecular features and survival by African and European ancestry in the cancer genome atlas. *JAMA Oncol* (2017) 3(12):1654. doi: 10.1001/jamaoncol.2017.0595

20. Marker KM, Zavala VA, Vidaurre T, Lott PC, Vásquez JN, Casavilca-Zambrano S, et al. Human epidermal growth factor receptor 2-positive breast cancer is associated with indigenous American ancestry in Latin American women. *Cancer Res* (2020) 80(9):1893–901. doi: 10.1158/0008-5472.CAN-19-3659

21. Serrano-Gómez SJ, Sanabria-Salas MC, Garay J, Baddoo MC, Hernández-Suarez G, Mejía JC, et al. Ancestry as a potential modifier of gene expression in breast tumors from Colombian women. *PloS One* (2017) 12(8):1–21. doi: 10.1371/journal.pone.0183179

22. Bivin WW, Yergiyev O, Bunker ML, Silverman JF, Krishnamurti U. GRB7 expression and correlation with HER2 amplification in invasive breast carcinoma. *Appl Immunohistochem Mol Morphol.* (2017) 25(8):553–8. doi: 10.1097/PAI.0000000000000349

23. Luoh S-W, Wagoner W, Wang X, Hu Z, Lai X, Chin K, et al. GRB7 dependent proliferation of basal-like, HER-2 positive human breast cancer cell lines is mediated in part by HER-1 signaling. *Mol Carcinog.* (2019) 58(5):699. doi: 10.1002/mc.22963

24. Nencioni A, Cea M, Garuti A, Passalacqua M, Raffaghello L, Soncini D, et al. Grb7 upregulation is a molecular adaptation to HER2 signaling inhibition due to removal of akt-mediated gene repression. *PloS One* (2010) 5(2):e9024. doi: 10.1371/journal.pone.0009024

25. Luoh S, Ramsey EE, Bai T, Keenan EJ. GRB7 and HER2 protein overexpression and breast cancer outcome. *J Clin Oncol* (2009) 27 (15_suppl):11102–2. doi: 10.1200/jco.2009.27.15_suppl.11102

26. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical Oncology/College of American pathologists clinical practice guideline update. *J Clin Oncol* (2013) 31(31):3997–4013. doi: 10.1200/JCO.2013.50.9984

27. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience* (2015) 4(1):7. doi: 10.1186/s13742-015-0047-8

28. Alexander DH, Novembre J, Lange K. Fast model-based estimation of ancestry in unrelated individuals. *Genome Res* (2009) 19(9):1655. doi: 10.1101/gr.094052.109

29. Sineshaw HM, Gaudet M, Ward EM, Flanders WD, Desantis C, Lin CC, et al. Association of race/ethnicity, socioeconomic status, and breast cancer subtypes in the national cancer data base (2010-2011). *Breast Cancer Res Treat* (2014) 145(3):753–63. doi: 10.1007/s10549-014-2976-9

30. Freedman RA, Virgo KS, He Y, Pavluck AL, Winer EP, Ward EM, et al. The association of race/ethnicity, insurance status, and socioeconomic factors with breast cancer care. *Cancer* (2011) 117(1):180–9. doi: 10.1002/cncr.25542

31. de Vries E, Buitrago G, Quitian H, Wiesner C, Castillo JS. Access to cancer care in Colombia, a middle-income country with universal health coverage. *J Cancer Policy.* (2018) 15:104–12. doi: 10.1016/j.jcpo.2018.01.003

32. Coronel E, Halstead D, Fregni F. Clinical research in Latin America: obstacles and opportunities. *Clin Investig (Lond).* (2011) 1(7):911–3. doi: 10.4155/cli.11.83

33. Ossa H, Aquino J, Pereira R, Ibarra A, Ossa RH, Pérez LA, et al. Outlining the ancestry landscape of Colombian admixed populations. *PloS One* (2016) 11 (10):e0164414. doi: 10.1371/journal.pone.0164414

34. Conley AB, Rishishwar L, Norris ET, Valderrama-Aguirre A, Mariño-Ramírez L, Medina-Rivas MA, et al. A comparative analysis of genetic ancestry and admixture in the Colombian populations of chocó and medellín. *G3 (Bethesda).* (2017) 7(10):3435–47. doi: 10.1534/g3.117.1118

35. AN H, JJ R, M R, DH G, ML C. HER2 status and disparities in luminal breast cancers. *Cancer Med* (2016) 5(8):2109–16. doi: 10.1002/cam4.757

36. Parise C, Caggiano V. Disparities in the risk of the ER/PR/HER2 breast cancer subtypes among Asian americans in California. *Cancer Epidemiol.* (2014) 38 (5):556–62. doi: 10.1016/j.canep.2014.08.001

37. Palmer JR, Ruiz-Narvaez EA, Rotimi CN, Cupples LA, Cozier YC, Adams-Campbell LL, et al. Genetic susceptibility loci for subtypes of breast cancer in an African American population. *Cancer Epidemiol Biomarkers Prev* (2013) 22 (1):127-34. doi: 10.1158/1055-9965.EPI-12-0769

38. Bryois J, Buil A, Evans DM, Kemp JP, Montgomery SB, Conrad DF, et al. Cis and trans effects of human genomic variants on gene expression. *PloS Genet* (2014) 10(7):e1004461. doi: 10.1371/journal.pgen.1004461

39. Geeleher P, Nath A, Wang F, Zhang Z, Barbeira AN, Fessler J, et al. Cancer expression quantitative trait loci (eQTLs) can be determined from heterogeneous tumor gene expression data by modeling variation in tumor purity. *Genome Biol* (2018) 19(1):130. doi: 10.1186/s13059-018-1507-0

40. Oak N, Cherniack AD, Mashl RJ, Carrot-Zhang J, Chambwe N, Damrauer JS, et al. Ancestry-specific predisposing germline variants in cancer. *Genome Med* (2020) 12(1):1–15. doi: 10.1186/s13073-020-00744-3

41. Fejerman L, Ahmadiyeh N, Hu D, Huntsman S, Beckman KB, Caswell JL, et al. Genome-wide association study of breast cancer in latinas identifies novel protective variants on 6q25. *Nat Commun* (2014) 5(1):1–8. doi: 10.1038/ncomms6260

42. Hoffman J, Fejerman L, Hu D, Huntsman S, Li M, John EM, et al. Identification of novel common breast cancer risk variants at the 6q25 locus among latinas. *Breast Cancer Res* (2019) 21(1):3. doi: 10.1186/s13058-018-1085-9

43. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast cancer. *Nat Rev Dis Primers* (2019) 5(1):66. doi: 10.1038/s41572-019-0111-2

44. Harbeck N, Gnant M. Breast cancer. *Lancet* (2017) 389(10074):1134–50. doi: 10.1016/S0140-6736(16)31891-8

45. PY C, YL T, TL S. Grb7, a critical mediator of EGFR/ErbB signaling, in cancer development and as a potential therapeutic Target. *Cells* (2019) 8(5):435. doi: 10.3390/cells8050435

46. Nadler Y, González AM, Camp RL, Rimm DL, Kluger HM, Kluger Y. Growth factor receptor-bound protein-7 (Grb7) as a prognostic marker and therapeutic target in breast cancer. *Ann Oncol Off J Eur Soc Med Oncol* (2010) 21(3):466–73. doi: 10.1093/annonc/mdp346

47. Prat A, Pascual T, Adamo B. Intrinsic molecular subtypes of HER2+ breast cancer. *Oncotar Impact Journ LLC;* (2017) 8:73362–3. doi: 10.18632/oncotarget.20629

48. Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Press M, et al. Adjuvant trastuzumab in HER2-positive breast cancer. *N Engl J Med* (2011) 365 (14):1273–83. doi: 10.1056/NEJMoa0910383

49. Batai K, Hooker S, Kittles RA. Leveraging genetic ancestry to study health disparities. *Am J Phys Anthropol.* (2021) 175(2):363–75. doi: 10.1002/ajpa.24144

50. Fejerman L, Romieu I, John EM, Lazcano-ponce E, Beckman KB, Pérez-stable EJ, et al. European Ancestry is positively associated with breast cancer risk in Mexican women. *Cancer Epidemiol Biomarkers Prev* (2011) 19(655):1074–82. doi: 10.1158/1055-9965.EPI-09-1193

51. Engmann NJ, Ergas IJ, Yao S, Kwan ML, Roh JM, Ambrosone CB, et al. Genetic ancestry is not associated with breast cancer recurrence or survival in U.S. latina women enrolled in the kaiser permanente pathways study. *Cancer Epidemiol Biomarkers Prev* (2017) 26(9):1466–9. doi: 10.1158/1055-9965.EPI-17-0148

52. Fejerman L, Ramirez AG, Napoles AM, Gomez SL, Stern MC. Cancer epidemiology in Hispanic populations: What have we learned and where do we need to make progress? *Cancer Epidemiol Biomarkers Prev* (2022) 31(5):932–41. doi: 10.1158/1055-9965.EPI-21-1303

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 04 January 2023
DOI 10.3389/fonc.2022.1091514

OPEN ACCESS

EDITED BY
Ariella Hanker,
University of Texas Southwestern Medical Center, United States

REVIEWED BY
Ewa Grzybowska,
Maria Skoldowska-Curie National Research Institute of Oncology, Poland
Gianluca Tedaldi,
Scientific Institute of Romagna for the Study and Treatment of Tumors (IRCCS), Italy

*CORRESPONDENCE
Nosheen Masood
dr.nosheen@fjwu.edu.pk

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 07 November 2022
ACCEPTED 30 November 2022
PUBLISHED 04 January 2023

CITATION
Khan I, Masood N and Yasmin A (2023) Correlation of ERCC5 polymorphisms and linkage disequilibrium associated with overall survival and clinical outcome to chemotherapy in breast cancer.
Front. Oncol. 12:1091514.
doi: 10.3389/fonc.2022.1091514

Correlation of ERCC5 polymorphisms and linkage disequilibrium associated with overall survival and clinical outcome to chemotherapy in breast cancer

Iqra Khan†, Nosheen Masood*† and Azra Yasmin†

Department of Biotechnology, Fatima Jinnah Women University, Rawalpindi, Pakistan

Purpose: ERCC5 is a DNA endonuclease and nucleotide excision repair gene; its mutations lead to a lack of activity by this enzyme, causing oxidative DNA damage. This study aimed to assess the role of four selected single nucleotide polymorphisms (SNPs) in ERCC5 and their linkage disequilibrium associated with survival analysis and clinical outcomes in breast cancer.

Patients and methods: Four SNPs (rs751402, rs17655, rs2094258, and rs873601) of the ERCC5 gene were analyzed using the PCR-RFLP technique, followed by sequencing in 430 breast cancer (BC) cases and 430 cancer-free individuals. Statistical analysis was performed using MedCalc 17 and SPSS version 24, while bioinformatic analysis of linkage disequilibrium was performed using Haploview software 4.2.

Results: Multivariate analysis showed that the rs751402 and rs2094258 polymorphisms were significantly associated with an elevated risk of BC ($P < 0.001$), while the other two SNPs, rs17655 and rs873601, did not show any association ($P > 0.001$). Survival analysis revealed that rs751402 and rs2094258 had longer overall survival periods ($P < 0.001$) than rs17655 and rs873601. Moreover, rs751402 and rs2094258 also had significantly longer overall survival (log-rank test, $P < 0.005$) for all three survival functions (positive family history, ER+PR status, and use of contraceptives), while rs17655 and rs873601 did not show any significant association. Only rs873601 showed a strong negative correlation with all the chemotherapeutic groups.

Conclusion: The current results suggest that variations in ERCC5 may contribute to BC development and that their genetic anomalies may be associated with cancer risk and may be used as a biomarker of clinical outcome.

KEYWORDS

breast cancer, ERCC5 gene, survival analyses, linkage disequilibrium, polymorphism

Introduction

Breast cancer (BC) is one of the most common malignancies and the primary cause of death among females worldwide (1). One in nine women in Pakistan faces this brutal disease (2). The mechanisms underlying breast carcinogenesis have not yet been fully explored and need to be completely understood. Various polymorphisms of genes involved in DNA damage responses play a significant role in cancer development and proliferation. Genes associated with DNA repair pathways are considered candidate genes for cancer susceptibility because reduced repair efficiency may induce carcinogenesis (3). One of the DNA repair pathways is the nucleotide excision repair (NER) pathway, which is significantly associated with cancer risk. Maintaining genomic stability and preventing the propagation of errors in the genome requires efficient DNA repair, and the NER pathway helps in the repair of bulky lesions such as thymine dimers generated by ultraviolet radiation (4). ERCC5 is a vital constituent of the NER mechanism and is called xeroderma pigmentosum group G (XPG). It encodes an endonuclease enzyme, which makes a structure-specific 3'- incision at damaged DNA sites. It can also act non-enzymatically by participating in a 5' incision with the help of the ERCC1/XPF heterodimer (5). ERCC5 is expressed in different tissues and cell lines, and its deficiency leads to genomic instability, DNA repair faults, and non-functioning gene transcription modulation and thus plays a role in DNA damage and higher breast cancer susceptibility, and regulation of DNA repair is a vital feature in various steps of carcinogenesis. Single nucleotide polymorphisms in ERCC5 may change its activity or expression, affecting DNA repair function, resulting in the alteration of cancer treatment effects, as treatment outcomes depend on the genetic variant of the gene present (6, 7).

Many studies have depicted that XPG polymorphisms are linked with various cancers like gastric, lung, breast, and colorectal (8–11). However, to our knowledge, only a limited number of studies have been conducted on the association analysis of these particular polymorphisms of ERCC5 (rs751402, rs17655, rs2094258, and rs873601) in BC patients and their response to chemotherapy. To investigate the possible influence of ERCC5 on BC, a case-control study was designed to evaluate the active involvement of these selected polymorphisms. Our study highlights the correlation of ERCC5 polymorphisms with various clinicopathological factors, overall survival rates with different survival functions, linkage disequilibrium analysis, and therapeutic outcomes of different chemotherapeutic drugs among breast cancer patients. Linkage disequilibrium analysis was conducted to explore the combined effects of these ERCC5 germline variants on breast carcinogenesis. It is expected that the data generated in the present study will help health practitioners make treatment decisions or provide the best advice based on an assessment of risk.

Materials and methods

Subjects and ethical considerations

The study was approved by the ethical committees of the Institute of Nuclear Medicine, Oncology, and Radiotherapy (INOR) Hospital, Abbottabad, Pakistan, and Fatima Jinnah Women University, Rawalpindi, Pakistan. The sample size was evaluated using a sample size calculator provided by the World Health Organization and validated manually by. Blood samples and demographic details were collected from 430 histologically confirmed breast cancer patients (mean age 47.32 ± 11.7) and healthy controls (mean age 46.3 ± 14.03, P = 0.005), with patients' consent signed by them to participate in the study (2019–2022). A questionnaire was designed for the collection of clinicopathological details of patients.

Single nucleotide polymorphism selection

Four potential SNPs (rs751402, rs17655, rs2094258, and rs873601) were selected from the National Center for Biotechnology Information SNP data base (http://www.ncbi.nlm.nih.gov/) and SNPinfo (http://snpinfo.niehs.nih.gov/) combined with previously described studies on the characteristics of the East Asian population in HapMap with minor allele frequency (MAF >5%). SNP rs17655 is a non-synonymous SNP (nsSNP) present in exon 15, while the remaining three SNPs are present in the regulatory region of ERCC5 (i.e., the 3′ untranslated region (UTR), the 5′ UTR promoter region, and the 5′ near gene). rs2094258 in the 5′ near gene was predicted to affect transcription factor binding site activity, rs751402 was present in the 5' UTR promoter region of the gene, and rs873601 in the 3'UTR may have an influence on the splicing and miRNA binding sites.

DNA extraction and polymorphism screening

Blood samples were collected in EDTA vacutainers and stored at −20°C until further use. Genomic DNA was isolated from blood samples by the standard phenol- chloroform method (5) and stored in a refrigerator at 4°C until further analysis. Qualitative analysis of DNA was performed using conventional electrophoresis on a 1% agarose gel and a spectrophotometer. Genotyping of the ERCC5 germline variants rs751402, rs17655, rs2094258, and rs873601 was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), following a method modified by Guo et al. (12). Primers were obtained from the published literature and are

listed in Supplementary Table S1 along with their respective references.

Statistical and survival analysis

Clinicopathological details, demographic characteristics, and ERCC5 variants between BC patients and healthy controls were analyzed using Pearson's chi- square test (χ^2) and Fisher's exact test. Conditional logistic regression was applied to find the associations between ERCC5 SNPs, clinicopathological details, and breast cancer risk by computing 95% confidence intervals (95% CIs) and odds ratios (ORs). Frequency distribution analysis was performed according to Hardy–Weinberg equilibrium (HWE) statistics. Patient follow- up was performed every 6 months and the homozygous wild variant was taken as a reference in all four ERCC5 SNPs. The overall survival (OS), survival distributions, and OS with three survival functions were estimated using Kaplan–Meier and log- rank tests. The survival distributions among the different classes of chemotherapy drugs were also assessed. Patients were classified based on the chemotherapeutic drugs administered. Taxanes, cytotoxic agents, and a combination of chemotherapeutic drugs were administered to all patients treated with chemotherapy. The frequency of chemotherapeutic drugs in breast cancer with both SNPs was analyzed by the chi- squared test. The correlation between SNPs and chemotherapeutic drugs was also assessed. Linkage disequilibrium analysis was performed using Haploview software 4.2. Significance level was set at $P < 0.05$. All statistical analyses were performed using IBM SPSS version 24 and MedCalc 17.

Results

Subject characteristics

The current study aimed to assess the genetic variations in the DNA excision repair protein ERCC-5 of the nucleotide excision repair pathway in 430 BC patients and 430 healthy controls. The demographic details and genotype frequencies of ERCC5 in patients with BC and healthy controls are shown in Table 1. The demographic parameters studied included family history, age, cancer staging, chemotherapeutic drug type,

TABLE 1 Frequency distribution of demographic factors, chemotherapeutic drugs, and ERCC5 germline variants in BC patients and controls.

Characteristics	Cases		Controls	
	Frequency	Percent	Frequency	Percent
Age groups				
15–30	30	3.5	97	11.3
31–45	172	20	314	36.5
46–60	177	20.6	328	38.1
61–85	51	5.9	121	14.1
Marital status				
Unmarried	82	95.3	4	4.7
Married	348	45	426	55
Family History				
No	381	56.3	296	43.7
Yes	49	26.8	134	72.2
Menopausal status				
Premenopausal	270	55.1	220	44.9
Postmenopausal	160	43.2	210	56.8
Chemotherapeutic drugs				
Cytotoxic	67	77.8		
Taxanes + Cytotoxic	134	15.6		
Cytotoxic + others	20	2.3		

(Continued)

TABLE 1 Continued

Characteristics	Cases		Controls	
	Frequency	Percent	Frequency	Percent
Taxanes + others	13	1.5		
Cytotoxic + Taxanes + others	2	0.2		
rs17655				
CC	343	79.8	407	94.7
CG	80	18.6	23	5.3
GG	7	1.6		
rs751402				
GG	66	15.3	323	75.1
AG	101	23.5	62	14.4
AA	263	61.2	45	10.5
rs2094258				
GG	200	46.5	269	62.6
AG	60	14	61	14.2
AA	107	39.5	100	23.3
rs873601				
AA	300	69.8	280	65.1
AG	80	18.6	70	16.3
GG	50	11.6	80	18.6

menopausal status, BMI, treatment type, marital status, and age at menarche. Most BC patients had stage III (39.4%) cancer, while only 16.2% had stage I cancer. Approximately 41.2% and 54.9% of the patients were treated with radiotherapy and chemotherapy, respectively. The data showed that the mean BMI for cases was 27.96 ± 5.64, showing obesity as a risk factor for BC (OR = 1.07, 95% CI = 2.62–3.65). Age, age at menopause, and menarche were evaluated as risk factors for BC (P <0.001). Significant differences were observed in marital status (OR = 0.4, 95% CI = 0.14–0.11, P <0.001), family history (OR = 0.28, 95% CI = 0.1–0.4, P <0.001), and menopausal status (OR = 1.61, 95% CI = 1.27–2.1, P <0.001) (Table 1).

Association of ERCC5 germline variants and clinicopathological parameters

To associate the genotype frequency of the assessed SNPs with clinicopathological factors, we applied logistic regression and $\chi 2$ tests. In this analysis, clinicopathological factors such as family history, marital status, ER status, PR status, and menopausal status were considered independent factors, and the genotype of all evaluated SNPs was considered a dependent variable, as illustrated in Table 2. The distribution frequency of the homozygous variant type and heterozygous variant type of ERCC5 rs751402 was only associated with patients who used

TABLE 2 Correlations between clinicopathological parameters and ERCC5 germline variants in patients with breast cancer (n = 430).

SNPs vs Parameters	Homozygous wild type No (%)	Variants types (homozygous, heterozygous) No (%)	P	OR (95% CI)	Z test
ERCC5 rs751402 vs					
Menopausal status	37 (56.1)	183 (50.3)	0.38	1.2 (0.7–2.1)	0.8
Premenopausal					

(Continued)

TABLE 2 Continued

SNPs vs Parameters	Homozygous wild type No (%)	Variants types (homozygous, heterozygous) No (%)	P	OR (95% CI)	Z test
Postmenopausal	29 (43.9)	181 (49.7)	0.3	0.79 (0.46–1.34)	0.86
Family History	18 (27.3)	114 (31.3)	0.5	0.8 (0.4–1.4)	0.65
Positive ER/PR status	45 (68.2)	282 (77.5)	0.1	0.6 (0.3–1.1)	1.6
Contraceptive use	25 (36.4)	63 (17.1)	0.0003	2.8 (1.6–4.9)	3.6
Married	64 (97)	361 (99.4)	0.1	0.2 (0.04–1.6)	1.4
ERCC5 rs17655 vs					
Menopausal status					
Premenopausal	206 (50.6)	14 (60.9)	0.3	0.6 (0.2–1)	0.95
Postmenopausal	201 (49.4)	9 (39.1)	0.4	0.65 (0.27–1.55)	0.9
Family History	121 (29.7)	11 (47.8)	0.07	2.1 (0.9–5.04)	1.79
Positive ER/PR status	311 (76.4)	16 (69.6)	0.4	0.7 (0.2–1.7)	0.7
Contraceptive use	84 (20.7)	2 (8.7)	0.1	0.3 (0.08–1.5)	1.4
Married	404 (99)	22 (100)	0.6	0.5 (0.02–9.5)	0.4
ERCC5 rs2094258 vs					
Menopausal status					
Premenopausal	102 (51)	118 (51.3)	0.9	0.9 (0.6–1.4)	0.06
Postmenopausal	98 (49)	112 (48.7)	0.9	1.15 (0.84–1.57)	0.9
Family History	61 (30.5)	71 (30.9)	0.9	1.19 (0.82–1.73)	0.94
Positive ER/PR status	153 (76.5)	174 (75.7)	0.8	1.23 (0.93–1.62)	1.47
Contraceptive use	42 (20.61)	45 (19.7)	0.7	1.07 (0.69–1.68)	0.33
Married	198 (1)	228 (99.1)	0.8	1.03 (0.79–1.35)	0.2
ERCC5 rs873601 vs					
Menopausal status					
Premenopausal	153 (51)	67 (51.5)	0.6	0.9 (0.6–1.3)	0.4
Postmenopausal	157 (49)	63 (48.5)	0.8	3.35 (2.4–4.66)	7.14
Family History	102 (34)	30 (23.1)	0.02	0.24 (0.15–0.37)	6.44
Positive ER/PR status	224 (74.7)	103 (79.2)	0.2	3.45 (2.57–4.61)	8.33
Contraceptive use	53 (17.7)	33 (25.6)	0.03	1.69 (1.07–2.67)	2.25
Married	297 (99.3)	128 (98.5)	0.47	5.26 (3.93–7.04)	11.2

contraceptives rather than the homozygous wild type (OR = 2.8; 95% CI = 1.6–4.9; P = 0.0003). The heterozygous and homozygous variant types of ERCC5 rs17655 were associated with patients who had a positive family history of cancer (OR = 2.1, 95% CI = 0.9–5.04; P = 0.05). There were no statistically significant correlations between the genotype distributions of both SNPs and menopausal, ER/PR, or marital status (Table 2). However, the analysis revealed a strong negative correlation between rs873601 and positive ER/PR status (OR = 3.45, 95% CI = 2.57–4.61; P = −0.04), rs873601 versus contraceptive use (OR = 1.69; 95% CI = 1.07–2.67; P = −0.09), and rs873601 versus menopausal status (OR = 3.35; 95% CI = 2.40–4.66; P = −0.005). No significant correlation was observed between rs873601 and family history (OR = 0.24; 95% CI = 0.15–0.37; P = 0.24), but married women showed a positive correlation (OR = 5.26; 95% CI = 3.93–7.04; P = 0.04) (Table 2). The fourth selected polymorphism rs2094258 showed a strong positive correlation with ER/PR status (OR = 1.23; 95% CI = 0.93–1.62; P = 0.01) and use of contraceptives (OR = 1.07, 95% CI = 0.69–1.68; P = 0.01), whereas a strong negative correlation was observed with marital status (OR = 1.03; 95% CI = 0.79–1.35; P = −0.007), family history (OR = 1.19; 95% CI = 0.82–1.73; P = −0.004), and menopausal status (OR = 1.15, 95% CI = 0.84–1.57; P = −003) (Table 2).

Linkage disequilibrium analysis

The analysis of linkage disequilibrium of the evaluated polymorphism of the ERCC5 gene was calculated using Haploview software, as shown in Figure 1. LD values are displayed as r2 and D values. Site 1 represents rs751402, site 2 represents rs17655, site 3 represents rs2094258 and site 4 represents rs873601. Sites 3 and 4 (rs2094258 and rs873601, respectively) exhibited a stronger association with LD among cancer patients than among healthy controls.

ERCC5 variants, survival distributions, and overall survival of breast cancer patients

Kaplan–Meier survival analysis and the log- rank test were used to determine the association between ERCC5 germline

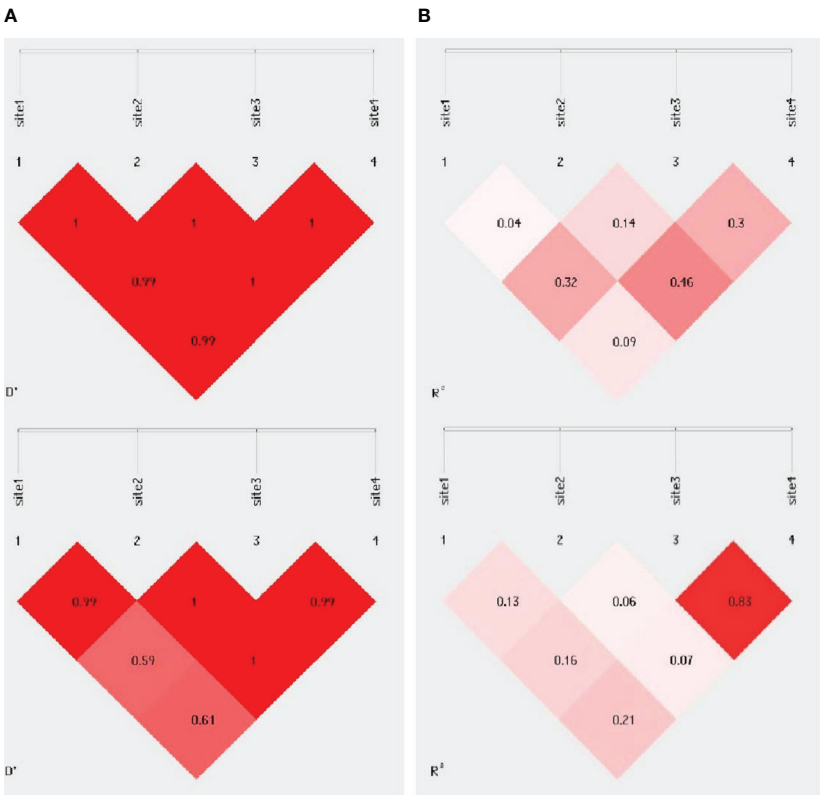


FIGURE 1
Pairwise linkage disequilibrium plot for evaluated ERCC5 polymorphism in **(A)** controls **(B)** BC cases. Site 1 is for rs751402, Site 2 is for rs17655, Site 3 for rs20942584 for rs873601. The darker area indicates higher D' & r^2 value.

variants and overall survival (OS) (Table S2). The rs751402 and rs2094258 polymorphism showed significant association (Log-rank test, P <0.001; Mean GG = 12, AA/AG = 22.3; 95% CI (GG) = 8.7–15.2, AA/AG = 20.8–23.8) (Log-rank test, P = 0.005; Median GG = 27 months, AG/AA = 21 months, 95% CI GG = 25.3–28.3, AA/AG = 16.4–25.5) (Figure S1) (Table S2), respectively, while the other two SNPs; rs17655 and rs873601, did not show any association with OS (Log-rank test, P = 0.3; Median CG + GG = 25; 95% CI = 20.3–29.6) (Log-rank test, P = 0.86; Median AA = 25 months, AG/GG = 26 months, 95% CI AA = 21.4–28.8, AA/AG = 16.4–35.5), respectively (Figure S1). Furthermore, we studied three survival functions: positive family history, ER/PR status, and contraceptive use, with all four evaluated SNP variants. It was found that rs751402 and rs2094258 had significantly longer OS for all three patient survival functions (log-rank test, P < 0.005). The estimated median for BC patients with a positive family history who had homozygous wild type rs751402 was 7 months (95% CI = 0.00–15.4), and for those with homozygous variant type and heterozygous variant type, it was 25 months (95% CI = 21.5–28.4) (Figure S1), and the estimated median having homozygous wild type with positive ER/PR status was 11 months (95% CI = 4.2–17.7), and for homozygous variant type and heterozygous variant type was 26 months (95% CI = 24.5–27.4) (Figure S1). For patients with rs751402 who had used contraceptives, the estimated median was 13 months (95% CI = 9.3–16.6) for homozygous wild type, while for homozygous variant type and heterozygous variant type it was 26 months (95% CI = 24.2–27.75) (Figure S1). No association was found for rs17655 and rs873601 with all three survival functions (log-rank test, P >0.5). For the patients with wild type for all three survival functions (positive family history, ER + PR status, and use of contraceptives) of rs17655, the median was 26 months (95% CI=19.2-32.7), 26 months (95% CI=21.5-30.4), and 25 months (95% CI=16.2-33.7), respectively, and for homozygous variant type and heterozygous variant type, the median was 21 months (95% CI = 15.1–26.8), 25 months (95% CI = 19.8–30.1), and 18 months (95% CI = 3.59–32.4), respectively (Figure S1). Similarly, for the patients with homozygous variant type and heterozygous variant type of rs873601, the median for all three survival functions (positive family history, ER + PR status, used contraceptives) was 26 months (95% CI = 0.00–59.3), 27 months (95% CI = 15.9-38), and 18 months (95% CI = 6.3–29.6), respectively, and for the wild type, the median was 25 months (95% CI = 21.1–28.8), 25 months (95% CI = 21.5–28.4), and 25 months (95% CI = 17.8–32.1) (Figure S1), respectively. For taking into consideration survival functions, evaluation for rs2094258 (positive family history, ER + PR status, used contraceptives), the median for homozygous and heterozygous variant types was 21 months (95% CI = 15.2–26.7), 24 months (18.3–29.6), and 18 months (95% CI = 11.43–24.5), respectively, while for the wild type was 27 months (95% CI = 24.7–29.2), 27 months (95% CI = 25.2–28.7), and 27 months (95% CI = 16.4–24.5), respectively (Figure S1).

Survival distributions for different chemotherapeutic drugs classes

Chemotherapeutic drug- related data were available for only 236 patients, possibly because they were not taking those drugs or had missing records from the files. We categorized all chemotherapeutic drugs given in different classes: cytotoxic drugs, taxanes, others, cytotoxic and taxanes, and all three were given together in combination. Patients were followed up every six months to inquire about their health condition, monitor the effectiveness of drugs, and for survival analysis. Genetic analysis was conducted to evaluate the association of SNPs with the response to a particular chemotherapeutic drug type. The outcomes are summarized in Table 3. We were unable to find any association between the respective chemotherapeutic drugs and rs17655 (P >0.001), whereas rs751402 showed a significant association (P <0.001). The overall survival for all the drugs administered was not statistically significant (log-rank test, P = 0.09). Survival differences for different drugs were compared through Breslow, Tarone–Ware, and the Log-rank test, which showed insignificant results for all drugs given (Log-rank test, P = 0.09; median = 18; 95% CI = 14.9–21.08) (Figure 2).

Discussion

The present study was designed to associate single nucleotide polymorphisms of ERCC5 (rs751402, rs2094258, rs17655, and rs873601) with breast cancer and associated risk factors. A significantly higher rate of variants at rs751402 and 2094258 was observed in breast cancer patients than in non-cancerous individuals, while the other two evaluated SNPs did not show any association. Only rs17655 was present in the exonic region, whereas the remaining three were present in the regulatory region of ERCC5. The present study reported elevated BC risk with a positive family history, showing similar results to previously reported literature (13, 14). The present study also reported that increased BC risk was linked to late menopause and early menarche, which is concordant with the literature (15). To maintain genome integrity, regulation of the NER pathway is essential, and ERCC5 is a multifunctional gene that encodes structure-specific endonucleases (16). Studies have found an association between ERCC5 genetic variations and different cancers (8). The current study reported a significant association between variant types of rs751402 and rs2094258 with an elevated risk of breast cancer. At present, very few studies are available with respect to the mentioned polymorphisms and breast cancer. Significant correlation of variant genotypes of rs751402, rs2094258, and rs873601 has been stated with colorectal cancer susceptibility (17). Pongsavee and Wisuwan (4) also reported a significant association of

TABLE 3 Genotyping frequency and correlation of ERCC5 germline variants with chemotherapeutic drugs in breast cancer patients.

SNPs vs drug type	Homozygous wild typeNo (%)	Variants types (homozygous, heterozygous) No (%)	Chi-Square (χ^2)	Pearson Correlation
ERCC5 rs751402 vs			P <0.001	0.2
Cytotoxic drugs	33 (53.3)	35 (19.9)		
Taxanes + Cytotoxic	19 (31.7)	115 (65.3)		
Cytotoxic + Others	9 (15)	11 (6.3)		
Taxanes + Others	0 (0)	13 (7.4)		
Cytotoxic + Taxanes + Others	0 (0)	2 (1.1)		
ERCC5 rs17655 vs			P >0.001	0.4
Cytotoxic drugs	63 (31.7)	4 (28.4)		
Taxanes + Cytotoxic	101 (50.8)	33 (89.2)		
Cytotoxic + Others	20 (10.1)	0 (0)		
Taxanes + Others	13 (6.5)	0 (0)		
Cytotoxic + Taxanes + Others	2 (1)	0 (0)		
ERCC5 rs2094258 vs			P = 0.1	P = 0.1
Cytotoxic drugs	39 (36.1)	28 (21.9)		
Taxanes + Cytotoxic	55 (50.9)	79 (61.7)		
Cytotoxic + Others	9 (8.3)	11 (8.6)		
Taxanes + Others	5 (4.6)	8 (6.3)		
Cytotoxic + Taxanes + Others	0 (0)	2 (1.6)		
ERCC5 rs873601 vs			P >0.001	−0.26
Cytotoxic drugs	32 (19.5)	35 (48.6)		
Taxanes + Cytotoxic	107 (65.2)	27 (37.5)		
Cytotoxic + Others	11 (6.7)	9 (12.5)		
Taxanes + Others	12 (7.3)	1 (1.4)		
Cytotoxic + Taxanes + Others	2 (1.2)	0 (0)		

rs751402 with breast cancer in a Thai population. Wang et al. (7) had described no association of rs17655 with BC among the Han population of northwest China and a significant association of rs751402 with breast carcinogenesis. A meta-analysis showed that rs873601 was significantly associated with overall risk, and another meta-analysis showed that this polymorphism is involved in the development and severity of colorectal cancer (8, 18). Guo et al. (12) investigated the role of rs17655 and rs751402 in the development of gastric cancer in a Chinese population and found that the mutant genotype of rs751402 significantly increased gastric cancer risk compared to the wild type, but rs17655 did not. Several meta-analyses have found that the rs17655 polymorphism might not confer susceptibility to breast cancer, and the results are still inconsistent (6, 19). Our study showed short survival due to delayed medical aid, a diverse medical history, and an advanced disease stage. Patients with early medical aid have higher 5- year survival rates than those with delayed presentation (88% and 12%, respectively) (20). Most of the patients in the present study had advanced stages of disease because they were from rural areas and mostly lacked disease knowledge and had financial constraints to go for therapeutic options and a good diet. Looking for medical aid early, before the advanced stage, implies a better prognosis and ultimately improves survival rates.

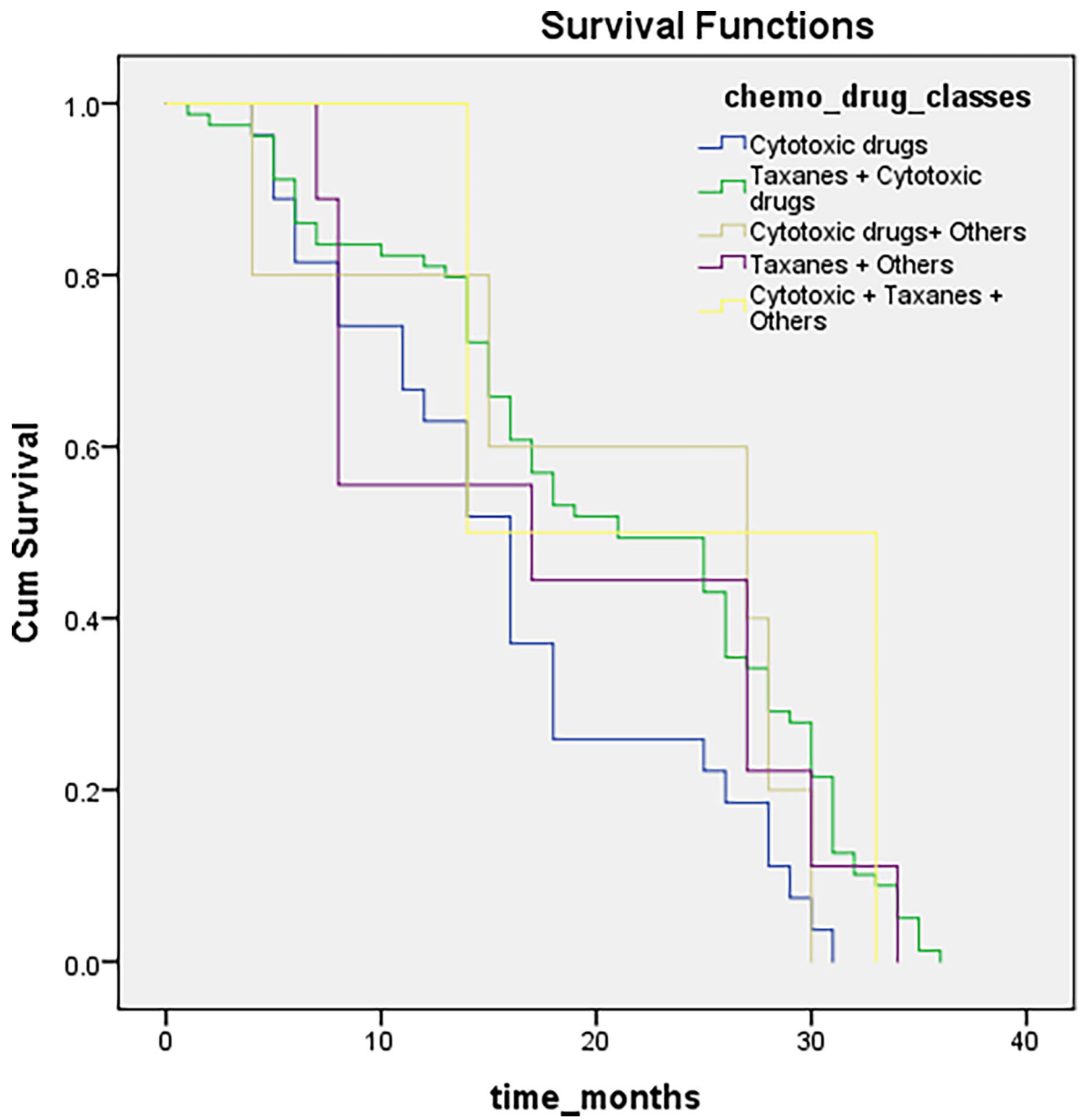


FIGURE 2
Illustrating survival distributions for different chemotherapeutic drugs classes among breast cancer patients.

Conclusion

In conclusion, two SNPs (rs751402 and 2094258) may play a role in the etiology of breast cancer in Pakistan. This is the first report of the association between ERCC5 (rs751402, rs2094258, rs17655, and rs873601) and breast cancer risk in Pakistan. The literature is limited in this area; therefore, for more pronounced results, studies with larger sample sizes are needed. Furthermore, late menopause, positive ER/PR status, and a positive family history are contributing factors to breast cancer development.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Fatima Jinnah Women University, Rawalpindi, Pakistan. The patients/participants provided their written informed consent to participate in this study.

Author contributions

IK was involved in the execution of research and experimental work. NM was involved in concept, design, write up. AY was involved in final draft review, experimental lab, and chemicals provision. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

The authors would like to thank the Higher Education Commission of Pakistan for funding this research under NRPU Project No. 7281.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1091514/full#supplementary-material

References

1. Baade P. Geographical variation in breast cancer outcomes *Int. J. Environ. Res. Public Health* (2017) 14(5):523. doi: 10.3390/ijerph14050523

2. Malik SS, Mubarik S, Aftab A, Khan R, Masood N, Asif M, et al. Correlation of MSH2 exonic deletions and protein downregulation with breast cancer biomarkers and outcome in Pakistani women/patients. *Environ Sci Pollut Res* (2021) 28(3):3066–77. doi: 10.1007/s11356-020-10717-z

3. Pongsavee M. Effects of ERCC5 rs751402 polymorphism on oxidative stress and the impact of curcumin on catalase activity in breast carcinogenesis. *Asian Pacific J Cancer Prev* (2022) 23(6):2065–70. doi: 10.31557/APJCP.2022.23.6.2065

4. Pongsavee M, Wisuwan K. ERCC5 rs751402 polymorphism is the risk factor for sporadic breast cancer in Thailand. *Int J Mol Epidemiol Genet* (2018) 9(4):27.

5. Malik SS, Mubarik S, Baig M, Masood N, Chaudhry N. Genetic polymorphism in ERCC5 and breast cancer risk. *Mol Biol Res Commun* (2019) 8 (1):27. doi: 10.22099/mbrc.2019.32294.1383

6. Xu Y, Jiao G, Wei L, Wang N, Xue Y, Lan J, et al. Current evidences on the XPG Asp1104His polymorphism and melanoma susceptibility: A meta-analysis based on case–control studies. *Mol Genet Genomics* (2015) 290(1):273–9. doi: 10.1007/s00438-014-0917-2

7. Wang H, Wang T, Guo H, Zhu G, Yang S, Hu Q, et al. Association analysis of ERCC5 gene polymorphisms with risk of breast cancer in han women of northwest China. *Breast cancer* (2016) 23(3):479–85. doi: 10.1007/s12282-015-0590-2

8. Han C, Huang X, Hua R, Song S, Lyu L, Ta N, et al. The association between XPG polymorphisms and cancer susceptibility: Evidence from observational studies. *Medicine* (2017) 96(32):32. doi: 10.1097/MD.0000000000007467

9. Huang J, Liu X, Tang L-L, Long JT, Zhu J, Hua RX, et al. XPG gene polymorphisms and cancer susceptibility: Evidence from 47 studies. *Oncotarget* (2017) 8(23):37263. doi: 10.18632/oncotarget.16146

10. Malik SS, Mubarik S, Masood N, Khadim MT. An insight into clinical outcome of XPG polymorphisms in breast cancer. *Mol Biol Rep* (2018) 45(6):2369–75. doi: 10.1007/s11033-018-4401-7

11. Su J, Zhu Y, Dai B, Yuan W, Song J. XPG Asp1104His polymorphism increases colorectal cancer risk especially in asians. *Am J Trans Res* (2019) 11 (2):1020

12. Guo B, Yang L, Zhao R, Hao S. Association between ERCC5 gene polymorphisms and gastric cancer risk. *Genet Mol Res* (2016) 15(2):15017510. doi: 10.4238/gmr.15027828

13. Pervaiz R, Tosun Ö, Besim H, Serakinci N. Risk factor assessment for breast cancer in north Cyprus: A comprehensive case-control study of Turkish Cypriot women. *Turkish J Med Sci* (2018) 48(2):293–304. doi: 10.3906/sag-1709-54

14. Aldabal BK, Koura MR. Risk factors of breast cancer among the primary health-care attendees in Eastern Saudi Arabia. *Int J Med Sci Public Health* (2016) 5 (2):276–81. doi: 10.5455/ijmsph.2016.16092015102

15. Dahiya N, Basu S, Singh MC, Garg S, Kumar R, Kohli C. Knowledge and practices related to screening for breast cancer among women in Delhi, India. *Asian Pacific J Cancer prevention: APJCP* (2018) 19(1):155. doi: 10.22034/APJCP.2018.19.1.155

16. Spivak G. Nucleotide excision repair in humans. *DNA repair* (2015) 36:13–8. doi: 10.1016/j.dnarep.2015.09.003

17. Hua R-X, Zhuo Z-J, Zhu J, Zhang SD, Xue WQ, Zhang JB, et al. XPG gene polymorphisms contribute to colorectal cancer susceptibility: A two-stage case-control study. *J Cancer* (2016) 7(12):1731. doi: 10.7150/jca.15602

18. Liang Y, Deng J, Xiong Y, Wang S, Xiong W. Genetic association between ERCC5 rs17655 polymorphism and lung cancer risk: Evidence based on a meta-analysis. *Tumor Biol* (2014) 35(6):5613–8. doi: 10.1007/s13277-014-1742-2

19. Aggarwal N, Donald ND, Malik S, Selvendran SS, McPhail MJ, Monahan KJ. The association of low-penetrance variants in DNA repair genes with colorectal cancer: a systematic review and meta-analysis. *Clin Trans gastroenterol* (2017) 8(7): e109. doi: 10.1136/gutjnl-2017-314472.247

20. Gulzar F, Akhtar MS, Sadiq R, Bashir S, Jamil S, Baig SM. Identifying the reasons for delayed presentation of Pakistani breast cancer patients at a tertiary care hospital. *Cancer Managment Res* (2019) 11:1087. doi: 10.2147/CMAR.S180388

frontiers | Frontiers in Oncology

TYPE Review
PUBLISHED 04 January 2023
DOI 10.3389/fonc.2022.1037531

OPEN ACCESS

EDITED BY
Ariella Hanker,
University of Texas Southwestern Medical Center, United States

REVIEWED BY
Angel Luis Guerrero-Zotano,
Instituto Valenciano de Oncologia, Spain
Jonathan T. Lei,
Baylor College of Medicine, United States
Alberto Servetto,
University of Naples Federico II, Italy

*CORRESPONDENCE
Rinath Jeselsohn
Rinath_Jeselsohn@dfci.harvard.edu
Zsuzsanna Nagy
Zsuzsanna_Nagy@dfci.harvard.edu

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 06 September 2022
ACCEPTED 22 November 2022
PUBLISHED 04 January 2023

CITATION
Nagy Z and Jeselsohn R (2023) ESR1 fusions and therapeutic resistance in metastatic breast cancer. *Front. Oncol.* 12:1037531. doi: 10.3389/fonc.2022.1037531

ESR1 fusions and therapeutic resistance in metastatic breast cancer

Zsuzsanna Nagy[1,2,3]* and Rinath Jeselsohn[1,2,3,4]*

[1]Center for Functional Cancer Epigenetics, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, United States, [2]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, United States, [3]Department of Medicine, Harvard Medical School, Boston, MA, United States, [4]Susan F. Smith Center for Women's Cancers, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, United States

Breast cancer is the most frequent female malignant tumor, and the leading cause of cancer death in women worldwide. The most common subtype of breast cancer is hormone receptor positive that expresses the estrogen receptor (ER). Targeting ER with endocrine therapy (ET) is the current standard of care for ER positive (ER+) breast cancer, reducing mortality by up to 40% in early- stage disease. However, resistance to ET represents a major clinical challenge for ER+ breast cancer patients leading to disease recurrence or progression of metastatic disease. Salient drivers of ET resistance are missense mutations in the ER gene (*ESR1*) leading to constitutive transcriptional activity and reduced ET sensitivity. These mutations are particularly prominent and deleterious in metastatic breast cancer (MBC). In addition to activating *ESR1* point mutations, emerging evidence imposes that chromosomal translocation involving the *ESR1* gene can also drive ET resistance through the formation of chimeric transcription factors with constitutive transcriptional activity. Although these *ESR1* gene fusions are relatively rare, they are enriched in ET resistant metastatic disease. This review discusses the characteristics of ER fusion proteins and their association with clinical outcomes in more aggressive and metastatic breast cancer. The structure and classification of ER fusion proteins based on function and clinical significance are also addressed. Finally, this review summarizes the metastatic phenotypes exhibited by the ER fusion proteins and their role in intrinsic ET resistance.

KEYWORDS

breast cancer, estrogen receptor, ESR1 fusion, endocrine therapy resistance, SERD

Introduction

Despite significant advances in breast cancer screening and treatment, mortality rates remain high with nearly 2.3 million new cases diagnosed and more than 650 000 patients dying each year worldwide according to the World Health Organization (1). The most common breast cancer subtype is hormone receptor positive, expressing the ER and/or progesterone receptor, accounting for approximately 75% of breast cancers (2). ER is a nuclear transcription factor that drives breast cancer development and growth. ER is comprised of four domains (3), an N-terminal activation function-1 (AF-1), a central DNA binding domain followed by a hinge region and the C-terminal ligand binding domain (LBD) that contains the ligand-dependent activation function (AF-2). Following estrogen binding, ER dimerizes and translocate to the nucleus where it binds to DNA at estrogen response elements (ERE) to regulate the transcription of multiple genes involved in tumor progression (3). ER functions as part of a transcriptional complex including (1) other transcription factors, such as Activator Protein 1 (AP1), Transcription Factor SP1 (SP1), Nuclear Factor-κB (NF-κB) and E2F Transcription Factor 1 (E2F1) (4–8); (2) co-factors that regulate chromatin structure, such as Nuclear Receptor Coactivator 1 (SRC-1), Nuclear Receptor Coactivator 2 (TIF2), Glutamate Receptor Interacting Protein 1 (GRIP-1), Amplified in Breast Cancer 1 (AIB1), CREB binding protein (CBP), p300 and the p300/CBP-associated factor (pCAF) (9–16); (3) pioneer factors that modulate ER binding to chromatin, such as Forkhead Box A1 (FOXA1), GATA Binding Protein 3 (GATA3), Pre-B-cell Leukemia Transcription Factor 1 (PBX1), and transducin-like enhancer protein 1 (TLE1) (17–23). ET is the mainstay treatment in ER+ breast cancer (24), and these treatment options include selective estrogen receptor modulators (SERMs), aromatase inhibitors (AI), selective estrogen receptor degraders (SERDs), and selective estrogen receptor covalent antagonists (SERCAs). SERMs, such as tamoxifen antagonize ER by reducing co-factor binding (25). AIs block the conversion of testosterone to estrogen and SERDs, such as fulvestrant, competitively bind ER and lead to proteasomal degradation (25). SERCA H3B-5942 inactivates ER by targeting Cys530 to enforce a distinct antagonist conformation (26, 27). Combination of ET with inhibitors for cyclin dependent kinase 4/6 (CDK4/6), mammalian target of rapamycin (mTOR), or phosphatidylinositol-4,5- biphosphate 3-kinase catalytic subunit alpha (PIK3CA) is integral part of the treatment in metastatic ER+ breast cancer and represent major improvements in progression free survival (28).

While ET reduces mortality by up to 40% in early-stage disease and highly effective in controlling metastatic disease, therapeutic resistance remains a momentous clinical issue (29, 30). At most, 20% of resistant cases lose ER expression (31) and in many patients the ER transcriptional axis remains active, however, in an altered fashion. To date, multiple mechanisms of acquired resistance to ET have been investigated and identified. These include (1) altered expression of transcription factors and co-regulatory proteins (e.g. SP1, AP1, NF-κB, SRC-1, AIB1, FOXA1) (18, 32–37), (2) modification of ER by miRNAs (e.g. miR-148, miR-152 and miR-221/222) (38–41), (3) increased crosstalk between ER/HER2/SRC3 (42), (4) amplification of tyrosine kinase receptors (e.g. fibroblast growth factor receptor 1 and 2 or insulin-like growth factor receptor 1) (43–46), (5) aberrant expression of cell cycle proteins (e.g. c-Myc, p21 and p27) (47–52) and (6) immune system-dependent resistance regulated *via* the NF-κB pathway (53) or chemokines activated PI3K/Akt/mTOR signaling (54, 55). Additional mechanism of ET resistance is the acquisition of somatic mutations in *ESR1* that are present in up to 50% of MBC patients (56–61). Widely studied examples are point mutations in the LBD (Y537S and D538G) that confer ER constitutive activity and exhibit decreased ET sensitivity (57, 59).

Studies have shown that chimeric proteins are powerful drivers of cancer with tremendous clinical impact (62). Larotrectinib, the first pan-cancer drug against the NTRK gene fusions demonstrated rapid responses in both adult and pediatric cancer patients (63–68). Driven by deep transcriptomic sequencing studies, several pathological gene fusions have been identified in aggressive (luminal B, basal like, or endocrine resistant breast cancer) breast cancers (69–72). These include fusion proteins associated with ER such as ESR1-CCDC170 (73–75), and ESR1-YAP1 (76) and non ER related fusions such as CTNNBL1-RAF1, ACTL6A-PIK3CA, S6KCI-AKT3 (71), SEC16A-NOTCH1 (77), SEC22B-NOTCH2 (72), and ETV6-NTRK3 (78). A number of these fusions promote tumor growth, and patients expressing these fusion proteins have more rapid disease progression and shorter survival than fusion-negative patients (70, 71, 75, 79). Identifying the full spectrum of the *ESR1* gene fusions and characterizing their role in intrinsic ET resistance is critical for developing novel and effective targeted therapies.

ESR1 fusions are acquired and enriched in MBC

RNA-seq analysis conducted by Veeraraghavan and colleagues on 990 primary TCGA breast samples identified the first *ESR1* gene fusion, ESR1-e2>CCDC170 (Table 1), in a subset (2.1%) of Luminal B breast tumor samples (75, 82). This fusion is formed by tandem-duplication, it retains the first two non-coding exons of *ESR1* (ESR1-e2) connected to various sequences from the coiled-coil domain containing 170 (CCDC170) gene (Figure 1). The promoter trap drives aberrant expression of CCDC170 and produces N-terminal truncated forms of the CCDC170 protein (ΔCCDC170) (75). The authors also provided functional evidence

TABLE 1 Summary of ESR1 gene fusions identified in breast cancers.

ESR1 fusion	Study cohorts and incidence	Clinical characteristics	Detection methods	Reference for detection	ESR1 break point exon	Frame	Functional fusion expression	Function	Reference for function
ESR1-CCDC170	cohort of 990 TCGA breast samples; 21 of 990 tumors (2.1%)	primary; luminal B subtype	RNA sequencing; PCR	(75)	exon 2	5'UTR-CDS	stable expression; produce truncated CCDC170 protein (rather than a chimeric protein)	increased cell migration and anchorage independent growth; increased colony-formation; reduced tamoxifen sensitivity	(75)
ESR1-YAP1	cohort of 22 patient-derived xenografts; 1 of 22 tumors	endocrine therapy resistant, metastatic ER +	RNA sequencing	(76)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; fulvestrant resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility and development of lung metastasis	(76) (80) (81)
ESR1-c6orf211/ARMT1	cohort of 15; 2 of 15 tumors	early stage (stage I-III) and non-metastatic ER+	RNA sequencing; Nano-string; PCR	(82)	exon 2	5'UTR-CDS	unknown	unknown	
ESR1-AKAP12	cohort of 15; 1 of 15 tumors	early stage (stage I-III) and non-metastatic ER+	RNA sequencing; Nano-string; PCR	(82)	exon 6	in-frame	unknown	unknown	
ESR1-ARNT2-e18	cohort of 91 breast cancer patients (MET500 cohort); 1 of 91	metastatic ER+	whole exome sequencing	(83)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; fulvestrant resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility	(81)
ESR1-PCMT1	cohort of 91 breast cancer patients (MET500 cohort); 1 of 91	metastatic ER+	whole exome sequencing	(83)	exon 6	in-frame	stable expression and inactive fusion protein	no role in estrogen independent and ET resistant growth	(81)

(Continued)

10.3389/fonc.2022.1037531

TABLE 1 Continued

ESR1 fusion	Study cohorts and incidence	Clinical characteristics	Detection methods	Reference for detection	ESR1 break point exon	Frame	Functional fusion expression	Function	Reference for function
ESR1-ARID1B	cohort of 91 breast cancer patients (MET500 cohort); 1 of 91	metastatic ER+	whole exome sequencing	(83)	exon 6	in-frame	stable expression and inactive fusion protein	no role in estrogen independent and ET resistant growth	(81)
ESR1-DAB2	cohort of 6 patient-matched breast cancer samples (University of Pittsburgh Health Science Tissue Bank); 1 of 6 tumors	supraclavicular lymph node metastasis; ER+	whole-genome sequencing RNA sequencing PCR immunoblot	(84)	exon 6	in-frame	stable expression and active context-dependent fusion protein	estrogen independent growth in MCF7 but not T47D cells	(81)
ESR1-GYG1	cohort of 6 patient-matched breast cancer samples (University of Pittsburgh Health Science Tissue Bank); 1 of 6 tumors	bone metastasis; ER+	whole-genome sequencing RNA sequencing PCR immunoblot	(84)	exon 6	in-frame	stable expression and inactive fusion protein	no role in estrogen independent and ET resistant growth	(81)
ESR1-SOX9	cohort of 9542 breast tumors (5216 from metastatic disease) from patients with advanced breast cancer (Foundation Medicine); 1 of 9542 (0.01%)	metastatic ER+ (solid tumor; liver metastasis)	comprehensive genomic profiling	(84)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; fulvestrant resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility	(84) (81)
ESR1-MTHFD1L	cohort of 9542 breast tumors (5216 from metastatic disease) from patients with advanced breast cancer (Foundation Medicine); 1 of 9542 (0.01%)	late-stage, endocrine-refractory (solid tumor; local reoccurrence)	comprehensive genomic profiling	(84)	exon 7	in-frame	unknown	unknown	
ESR1-PLEKHG1	cohort of 9542 breast tumors (5216 from metastatic disease) from patients with advanced breast cancer (Foundation Medicine); 1 of 9542 (0.01%)	late-stage, endocrine-refractory, metastatic (solid tumor; liver metastasis)	comprehensive genomic profiling	(84)	exon 6	in-frame	unknown	unknown	
ESR1-TFG	cohort of 9542 breast tumors (5216 from metastatic disease) from patients with advanced	late-stage, endocrine-refractory, metastatic (solid tumor; liver metastasis)	comprehensive genomic profiling	(84)	exon 6	in-frame	unknown	unknown	

(Continued)

10.3389/fonc.2022.1037531

TABLE 1 Continued

ESR1 fusion	Study cohorts and incidence	Clinical characteristics	Detection methods	Reference for detection	ESR1 break point exon	Frame	Functional fusion expression	Function	Reference for function
	breast cancer (Foundation Medicine); 1 of 9542 (0.01%)		RNA sequencing						
ESR1-NKAIN2	cohort of 254 ctDNA samples from patients with advanced breast cancer (Foundation Medicine); 1 of 254 tumors (0.39 %)	Stage IV, endocrine refractory (ctDNA)	comprehensive genomic profiling	(84)	exon 6	in-frame	unknown	unknown	
ESR1-CDK13	cohort of 254 ctDNA samples from patients with advanced breast cancer (Foundation Medicine); 1 of 254 tumors (0.39 %)	Stage IV, endocrine refractory (ctDNA)	comprehensive genomic profiling	(84)	exon 7	in-frame	unknown	unknown	
ESR1-COA5	cohort of 110 advanced ER + breast cancer patients (Clinical Genotyping Cohort); 1 of 110 (0.9%)	metastatic ER+	anchored multiplex PCR	(71)	exon 4	in-frame	unknown	unknown	
ESR1-PCDH11X	cohort of 25 breast cancers	late stage, endocrine-refractory, metastatic ER+	RNA sequencing	(80)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; fulvestrant resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility and development of lung metastasis	(80) (81)
ESR1-NOP2	cohort of 81 primary breast cancers [neoadjuvant AI (NeoAI) Trials]; 1 of 81	primary, treatment-naive, ER+	RNA sequencing	(80)	exon 6	in-frame	stable expression and inactive fusion protein	no role in estrogen independent and ET resistant growth	(80)
ESR1-AKR1D1	cohort of 81 primary breast cancers [neoadjuvant AI (NeoAI) Trials]; 1 of 81	primary, treatment-naive, ER+	RNA sequencing	(80)	exon 6	out-of-frame	N/A	N/A	
ESR1-POLH	cohort of 728 TCGA breast tumors; 1 of 728 (0.13%)	primary	RNA sequencing PCR	(80)	exon 7	in-frame	stable expression and inactive fusion protein	no role in estrogen independent and ET resistant growth	(80)
ESR1-CCDC170	cohort of 728 TCGA breast tumors; 1 of 728 (0.13%)	primary	RNA sequencing	(80)	exon 4	out-of-frame	N/A	N/A	

(Continued)

10.3389/fonc.2022.1037531

TABLE 1 Continued

ESR1 fusion	Study cohorts and incidence	Clinical characteristics	Detection methods	Reference for detection	ESR1 break point exon	Frame	Functional fusion expression	Function	Reference for function
ESR1-CCDC170	cohort of 728 TCGA breast tumors; 1 of 728 (0.13%)	primary	RNA sequencing	(80)	exon 5	out-of-frame	N/A	N/A	
ESR1-ARNT2-e2	cohort of 2520 pairs of tumor and normal tissues (The Hartwig Medical cohort)	metastatic ER+	whole-genome sequencing	(85)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; ET resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility	(81)
ESR1-LPP	cohort of 2520 pairs of tumor and normal tissues (The Hartwig Medical cohort)	metastatic ER+	whole-genome sequencing	(85)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; ET resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility	(81)
ESR1-NCOA1	cohort of 2520 pairs of tumor and normal tissues (The Hartwig Medical cohort)	metastatic ER+	whole-genome sequencing	(85)	exon 6	in frame	stable expression and active fusion protein	estrogen independent growth; ET resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility	(81)
ESR1-TCF12	cohort of 2520 pairs of tumor and normal tissues (The Hartwig Medical cohort)	metastatic ER+	whole-genome sequencing	(85)	exon 6	in-frame	stable expression and inactive fusion protein	no role in estrogen independent and ET resistant growth	(81)
ESR1-CLINT1	cohort of 2520 pairs of tumor and normal tissues (The Hartwig Medical cohort)	metastatic ER+	whole-genome sequencing	(85)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; ET resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility	(81)
ESR1-GRIP1	cohort of 2520 pairs of tumor and normal tissues (The Hartwig Medical cohort)	metastatic ER+	whole-genome sequencing	(85)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; ET resistant growth; constitutive expression	(81)

(Continued)

10.3389/fonc.2022.1037531

TABLE 1 Continued

ESR1 fusion	Study cohorts and incidence	Clinical characteristics	Detection methods	Reference for detection	ESR1 break point exon	Frame	Functional fusion expression	Function	Reference for function
								of ER and EMT-like transcriptional programs; increased cell motility	
ESR1-TNRC6B	cohort of 2520 pairs of tumor and normal tissues (The Hartwig Medical cohort)	metastatic ER+	whole-genome sequencing	(85)	exon 6	in-frame	stable expression and active fusion protein	estrogen independent growth; ET resistant growth; constitutive expression of ER and EMT-like transcriptional programs; increased cell motility	(81)

Study cohorts, incidence (for patient cohort > n=100), clinical characteristics, identification sites and validation methods are detailed. Functional ESR1 domains preserved in the gene fusion, in frame expression and function of ESR1 fusions are also noted.

that this fusion promotes more aggressive oncogenic phenotypes in ER+ breast cancer cells, such as increased cell migration, invasion, and reduced tamoxifen sensitivity. Utilizing RNA sequencing, Li and colleagues (76) described the first inter-chromosomal ESR1 fusion in a patient-derived xenograft from ET resistant MBC (Luminal A subtype, skin metastasis). This fusion is formed by a translocation event that brought *ESR1* exons 1 to 6 (ESR1-e6) on chromosome (chr) 6q into the yes associated protein 1 gene *YAP1* locus on chr11q (ESR1- e6>YAP1; Table 1), replacing the LBD of ESR1 with the transactivation domain (TAD) sequence from YAP1 (Figure 1). Although Li and colleagues conducted limited functional studies, overexpression of ESR1-e6>YAP1 in ER+ breast cancer cells conferred estradiol-independent growth in their study (Table 1). Lei and colleagues (80) and Gou and colleagues (81) provided additional mechanistic data for the ESR1-e6>YAP1 fusion and described its functional properties in driving estrogen-independent growth, constitutive expression of ER target genes, and anti-estrogen resistance. Several years later, two additional ESR1 fusions, ESR1-e2 fusion with the acidic residue methyltransferase 1 gene, C6orf211/ARMT1 (ESR1-e2>C6orf211/ARMT1) and ESR1-e6 fusion with a-kinase anchoring protein 12 gene, AKAP12 (ESR1-e6>AKAP12) were identified in AI resistant breast cancer by Giltnane and colleagues (Table 1) (82) with no functional data available (Table 1). Using whole genome sequencing, Robinson and colleagues (83) identified three additional ESR1 fusions (Table 1) including fusionsaryl hydrocarbon receptor nuclear translator 2 gene, ARNT2 (ESR1-e6>ARNT2-e18); protein-L-isoaspartate O-methyltransferase gene, PCMT1 (ESR1-e6>PCMT1); AT-rich interaction domain 1B gene, ARID1B (ESR1-e6>ARID1B), but the functional properties of these fusions were investigated only later on by Gou and colleagues (81). Hartmaier and colleagues also described the expression of ESR1-e6>AKAP12 in ER+ MBC

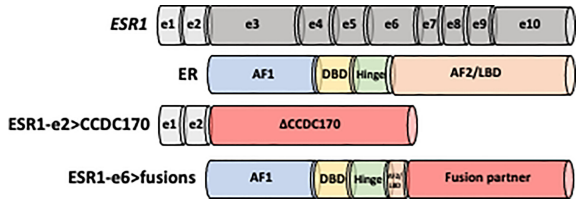


FIGURE 1
Schematic structure of ESR1-e2>CCDC170 and ESR1-e6>fusion proteins. Non-coding exons (e) 1 and 2 are shown as white boxes, while encoding domains in the ESR1 codon structure are presented in gray. ER is comprised of four domains: N-terminal activation function-1 (AF-1), DNA binding domain (DBD), hinge region and C-terminal ligand binding domain (AF-2/LBD). ESR1-e2>CCDC170 fusion proteins retain the first two non-coding exons of *ESR1* (ESR1-e2) and link to the coiled-coil domain containing 170 (CCDC170) gene generating truncated CCDC170 proteins (ΔCCDC170). ESR1-e6>fusions preserve the first 6 exons of *ESR1* (ESR1-e6), while replace the LBD by a 3' fusion partner.

and identified several novel ESR1 fusions (84). The authors also established that ESR1 fusion proteins are enriched in ER+ MBC (Table 1) and contribute to ET resistance. Hartmaier and colleagues described eight novel ESR1 fusion proteins (Table 1), all with junctions between ESR1 exon 6 and 7. ESR1-e6>fusions included DAB adaptor protein 2 gene, DAB2 (ESR1-e6>DAB2); glycogenin-1 gene, GYG (ESR1-e6>GYG1); SRY-box transcription factor 9 gene, SOX9 (ESR1-e6>SOX9); pleckstrin homology and RhoGEF domain containing G1, PLEKHG1 (ESR1-e6>PLEKHG1); trafficking from ER to Golgi regulator, TFG (ESR1-e6>TFG); and sodium/potassium transporting ATPase interacting 2, NKAIN2 (ESR1-e6>NKAIN2). ESR1-e7>fusions included mitochondrial isozyme of C1-tetrahydrofolate (THF) synthase, MTHFD1L (ESR1-e7>MTHFD1L) and cyclin dependent kinase 13, CDK13 (ESR1-e7>CDK13). The authors emphasized that many genetic rearrangement events are not expressed or translated into functional protein products, therefore they utilized an array of techniques (DNA and/or RNA sequencing, PCR and immunoblot) to investigate fusion protein expression (Table 1). As example, ESR1-e6>DAB2 and ESR1-e6>GYG1 were detected by DNA and/or RNA sequencing, and immunoblot, and *in vitro* studies established that these fusions were stable and active. The authors were able to detect ESR1-e6>SOX9, ESR1-e7>MTHFD1L, ESR1-e6>PLEKHG1, ESR1-e6>NKAIN2, ESR1-e6>AKAP12, and ESR1-e7>CDK13 only by DNA sequencing with low confidence in producing fusion transcripts. Utilizing anchored multiplex PCR, Matissek and colleagues (71) identified an additional ESR1 fusion protein (Table 1) with junction between ESR1 exon 4 and 3' fusion partner cytochrome C oxidase assembly factor 5, COA5 (ESR1-e4>COA5). The role of this fusion in MBC and ET resistance is currently unknown. Lei and colleagues (80) conducted a comprehensive study and identified several novel *ESR1* fusions (Table 1). The protocadherin 11 X-linked fusion (ESR1-e6>PCDH11X) was identified from a male patient with ER + MBC. Inter-chromosomal *ESR1* translocations included the nucleolar protein 2 homolog gene, NOP2 (ESR1-e6>NOP2), and aldo-keto reductase family 1 member D1 (ESR1-e6>AKR1D1). Fusion with DNA polymerase eta gene, POLH (ESR1-e7>POLH) was formed by intra-chromosomal translocation. In this study, the authors also identified the ESR1-e4>CCDC170 and ESR1-e5>CCDC170 fusions. Lei and colleagues conducted functional studies and provided further evidence that ESR1-e6>YAP1 and ESR1-e6>PCDH11X, identified from ER+ MBC, encoded stable and functional fusion proteins and promoted estrogen-independent growth, induced cellular motility, constitutive expression of ER target genes, and anti-estrogen resistance. Seven additional ESR1-e6>fusions (Table 1) were identified by Priestley and colleagues in ER+ MBC, including aqryl hydrocarbon receptor nuclear translocator 2, ARNT2 (ESR1-e6>ARNT2); LIM domain containing preferred translocation partner in lipoma, LPP (ESR1-e6>LPP); nuclear receptor coactivator 1, NCOA1 (ESR1-e6>NCOA1); transcription factor 12, TFC12 (ESR1-e6>TCF12); clathrin interactor 1, CLINT1 (ESR1-e6>CLINT1); glutamate receptor interacting protein 1, GRIP1 (ESR1-e6>GRIP1) and trinucleotide repeat containing adaptor 6B, TNRC6B (ESR1-e6>TNRC6B). Functional characterization of these fusions were investigated by Gou and colleagues (81, 85) (Table 1). Except ESR1-e6>TCF12, all ESR1-e6>fusions promoted estrogen-independent growth. It is noteworthy that up to date, only few ESR1 fusions (ESR1-e2>CCDC170, ESR1-e4>CCDC170, ESR1-e5>CCDC170, ESR1-e6>NOP2, ESR1-e6>AKR1D1, ESR1-e6>POLH) were detected in primary breast cancer samples (Table 1).

Structure and function of ESR1-e6>fusion proteins in MBC

The ESR1-e2>CCDC170 fusion protein consists of the 5′ untranslated region of ESR1 to the coding region of CCDC170, generating N-terminally truncated CCDC170 proteins (ΔCCDC170) expressed under the *ESR1* promoter (Figure 1) (75, 82). Structural studies have revealed that this structure is distinct from the ESR1-e6>fusions identified from ET resistant MBC. Despite the diversity among the ESR1-e6>fusions, they share a common structure whereby the first 6 exons of *ESR1* (ESR1-e6) are preserved, retaining the hormone-independent transactivation domain (TAD) as well as the DNA-binding domain of ER whereas the LBD is lost and replaced with a functional domain of the 3' fusion partner (Figure 1) (76, 81, 84). This structure is strongly associated with estrogen independent growth and ET resistant metastatic ER+ breast tumors. The loss of a functional LBD suggests a clear pathological impact, leading to complete resistance to the activity of current ER antagonists, which all bind to the LBD. As expected, several ESR1-e6>fusions (ESR1-e6>YAP1, ESR1-e6>PCDH11X, ESR1-e6>SOX9 and ESR1-e6>ARNT2-e18) remained stably expressed in the presence of fulvestrant and promoted ET-resistant growth of T-47D and MCF7 cells (80, 81). In contrast, the expression of ER mutant constructs that lack the LBD had decreased transcriptional activity, suggesting that the presence of the 3' partner is essential for the ER fusion activity (80, 81, 84). The fact that multiple different 3' partners have the same effect and drive ET resistance and malignant phenotypes, indicates that the enhanced activity of the ER fusions is not dependent on a specific 3' partners. These findings suggest that the 3' partner may be important for the stability of ER and possibly the dimerization of ER, however, an intact LBD is not required for ER activity in the context of the ER fusions.

It is important to note that not all ESR1-e6>fusions produce stable proteins with clear transcription factor (TF) or co-activator (CoA) functions, and only a subset of the ESR1-e6>fusions are activating fusions. The number of studies investigating the activity

of ESR1-e6>fusions is limited, the function of some fusions are still unknown. Further studies are required to investigate and fully validate the stability and activity of ESR1-e6>fusions. Some ESR1-e6>fusions such as ESR1-e6>YAP1, ESR1-e6>SOX9, ESR1-e6>ARNT2, ESR1-e6>LPP, ESR1-e6>NCOA1, ESR1-e6->PCDH11X, ESR1-e6>CLINT1, ESR1-e6>GRIP1 and ESR1-e6>TNRC6B produce stable and active fusion proteins that are positive regulators of transcription (80, 81). ESR1-e6->DAB2 has cell type specific transcriptional activity- active in MCF7 but not T47D cells. In contrast to transcriptionally active ESR1-e6>fusions, multiple ESR1- e6>fusions (e.g. ESR1-e6>TCF12, ESR1-e6>ARID1B, ESR1-e6>NOP2) were identified as transcriptionally inactive despite producing stable fusion protein, adding to the complex landscape of ESR1- e6>fusion proteins. None of the 3' partner genes of these latter ESR1-e6>fusions are known to be a TF or CoA and the wild-type protein is not nuclear-localized (80, 81). As hypothesized by Gou and colleagues (81), these transcriptionally inactive ESR1-e6>fusions could (1) encode tumor suppressors, (2) be active exclusively in the presence of a specific set of coactivators, or (3) act as dominant negative regulators interrupting the function of the remaining intact wild-type protein activity.

Activating ESR1 fusion proteins drive endocrine resistance and metastatic phenotypes

When first described, ESR1-e2>CCDC170 in ER+ breast cancer cells led to enhanced growth and reduced sensitivity to tamoxifen (75) suggesting a role for ESR1-e2>CCDC170 in ET resistance. Additional pre-clinical studies (74, 75, 79) showed that the expression of ESR1-e2>CCDC170 fusions in ER-positive breast cancer cells resulted in increased cell migration, increased colony formation, and increased cell proliferation as evidenced by the increase in the number of cells in S-G2/M phase. Li and colleagues (74) provided detailed evidence supporting the function of ESR1-e2>CCDC170 in promoting breast cancer cell survival and endocrine resistance both *in vitro* and in xenograft models. Their mechanistic study suggests that ESR1-e2>CCDC170 fusions bind and stabilize the HER2/HER3/SRC complex and enhance the activation of SRC/PI3K/AKT signaling during ET *in vitro* and *in vivo*. This study also suggested a potential strategy to manage ESR1-e2>CCDC170 positive patients by combining the HER2 inhibitor lapatinib and/or SRC inhibitor dasatinib with ET.

A series of publications clearly demonstrated that ESR1-e6>fusions were identified from ER+ MBC patients and most ESR1-e6>fusion proteins are drivers of ET resistance (69, 76, 80, 81, 84). Functional properties of these ESR1-e6>fusions include estradiol-independent growth and constitutive expression of ER target genes leading to ET-resistant proliferation and epithelial-mesenchymal transition (EMT) genes facilitating metastasis. The latest and most comprehensive study by Gou and colleagues (81) functionally screened multiple ESR1-e6>fusions and 4 were found to promote estradiol-independent cell growth, migration, EMT and resistance to fulvestrant. The ESR1-e6>YAP1, ESR1-e6>PCDH11X, ESR1-e6>SOX9, and ESR1-e6>ARNT2-e18 fusions promoted cell proliferation and migration in a hormone-independent and fulvestrant-resistant manner in multiple ER+ cell models. Although the four other ESR1-e6>fusions included in this study (ESR1-e6>DAB2, ESR1-e6>GYG1, ESR1-e6>PCMT1, and ESR1-e6>ARID1B) produced stable proteins, they did not promote ET-resistant growth. Moreover, RNA-seq showed that ER-positive breast cancer cells expressing ESR1-e6>YAP1, ESR1-e6>PCDH11X, ESR1-e6>SOX9 and ESR1-e6>ARNT2-e18 fusions upregulated the same cluster of ER target genes that were observed in the control cells stimulated by estradiol and drove constitutive expression of these ER target genes in the absence of estrogen. Pathway analysis also revealed that these transcriptionally active ESR1-e6>fusion proteins upregulated two EMT-related genes, SNAI1 (Snail) and VCAN (versican). The other ESR1-e6>fusions (ESR1-e6>GYG1, ESR1-e6>PCMT1, and ESR1-e6>ARID1B) did not induce estradiol-independent activation of ER and EMT target genes, despite the fact that they translocated to the nucleus. Additional functional studies showed the transcriptionally active ESR1-e6>YAP1 and ESR1-e6>PCDH11X fusions induced cell motility *in vitro* and promoted metastasis to the lung in cell-line xenograft models as well as in a PDX model harboring the ESR1-YAP1 fusion.

ESR1 fusions as potential biomarkers and novel therapeutic vulnerabilities in breast cancer

Next generation sequencing (NGS) methods such as DNA-based comprehensive whole genome (WGS) or RNA-based transcriptome (WTS) sequencing have been extensively used to describe gene fusions in multiple cancer types (86–90). WGS detects gene fusions based on hybrid-capture methods and still considered the most unbiased approach to identify fusion events, especially in large gene panels. WGS is highly sensitive and can be used on fresh, snap frozen and formalin fixed specimens. Still and all, WGS does not indicate the expression of the gene fusions, and the detection of fusion variants involving large DNA intronic regions is poor (86, 91). WTS have the overall advantage of detecting transcriptionally expressed gene fusions and the sequencing is not affected by intronic regions. Moreover, WTS does not require *a priori* knowledge of gene fusion partners, can distinguish splicing isoforms, quantify fusion transcripts, and it requires low input material. WTS approaches can be based on hybrid-capture or amplicon-based methods using classical or anchor multiplex PCR (86, 92–94).

Matissek and colleagues reported anchored multiplex PCR (AMP) as an effective approach to identify gene fusions in cancer, including ER+ metastatic breast cancer (71). AMP was also validated in their study and applied to cohorts of (1) 110 of early-stage and advanced ER+ breast cancer patients (Clinical Genotyping Cohort) and (2) 63 of advanced ER+ breast cancer patients with matched primary and metastatic samples (Matched Primary/Metastasis Cohort). 14 patients in the Clinical Genotyping Cohort harbored intergenic exon–exon fusions, including the in-frame fusion of *ESR1* to *CCDC170*. The authors emphasized that the identified fusion junction sequences involved at least one precise exon boundary. 10 of 63 patients in the Matched Primary/Metastasis Cohort harbored gene fusions in either the primary or metastatic samples. Collectively, AMP detected fusions in 24 of 173 breast cancer patients (14%) in this study, including 11 primary tumors. Combined with complementary "break-apart" fluorescence *in situ* hybridization (FISH) analysis, they further validated AMP as detection technique for clinically relevant fusions. As example, break-apart of *ESR1* was present in primary tumors and metastases from a patient whose tumors demonstrated the ESR1-e4>COA5 fusion upon AMP analysis. Additionally, the ESR1-e2>*CCDC170* fusion detected by AMP was also confirmed upon FISH analysis for *ESR1*. A disadvantage of WTS is that it only identifies expressed fusion genes and not adequate for gene fusion analysis at DNA level. Moreover, biological material is often short, resulting in poor quality RNA and false positive sequencing results. For multiplex PCR approach, the primer design and PCR bias like allele dropout can also impact analysis result (86, 92–94).

ESR1-e6>fusion genes have been detected by NGS methods (Table 1), techniques that are not yet employed routinely in the clinic. Analysis of plasma circulating tumor DNA to detect *ESR1* point mutations by droplet digital PCR (ddPCR) is now done in the clinic and clinical trials are investigating the use of these assays in real-time (95–97). Obtaining circulating DNA from liquid biopsies conserves the genomic landscape of the tumor suggesting that this less invasive detection methods may efficiently identify ESR1 fusions, particularly in metastatic ET resistant ER+ breast cancer. Indeed, Hartmaier and colleagues (84) used this approach to provide additional evidence of ESR1 fusion recurrence following extensive ER-targeted endocrine therapies. They obtained target capture sequencing data and examined a cohort of 9542 solid breast tumors and a cohort of 254 ctDNA samples from patients with advanced breast cancer. They successfully identified the ESR1-e6>SOX9, ESR1-e7>MTHFD1L, ESR1-e6>PLEKHG1, and ESR1-e6>TFG fusions (Table 1) in four solid tumors and the ESR1-e6>NKAIN2, ESR1-e6>AKAP12, and ESR1-e7>CDK13 (Table 1) fusions in 3 ctDNA samples. While there are several commercial platforms available and the cost of ctDNA assay is acceptable in the clinic, the concentration of ctDNA in plasma correlates with tumor size and stage, thus this assay is likely to be useful for late-stage breast cancer patients only (98).

As aforementioned, several ESR1-e6>fusion proteins are inactive and therefore not clinically actionable. As a potential efficient approach for screening samples for the presence of ESR1 fusions that drive ET failure in MBC, Gou and colleagues (81) developed a 24-gene expression signature that is specific for the presence of transcriptionally active ESR1 fusion proteins. Specifically, they identified 24 Hallmark genes, including 19 genes in the estrogen response gene set (CHST8, MAPT, OLFM1, PDZK1, RASGRP1, MPPED2, GREB1, MYB, GFRA1, PGR, ELOVL2, ADCY1, NPY1R, TFF1, ACOX2, SGK1, STC2, CALCR and KRT13), two genes in the EMT gene set (VCAN and COL3A1), and three genes shared in both gene sets (CXCL12, GJA1 and TGM2). To compare the transcriptional profile of ESR1-e6>fusions with known activating *ESR1* LBD point mutations (Y537S and D538G), Gou and colleagues performed RNA-sequencing on T47D cells that overexpressed either several ESR1 fusion proteins (ESR1-e6>ARNT2-e2, ESR1-e6>LPP, ESR1-e6>NCOA1, ESR1-e6>CLINT1, ESR1-e6>TNRC6B and ESR1-e6>GRIP1), or the Y537S and D538G point mutations. ESR1-e6>ARNT2-e2, ESR1-e6>LPP, ESR1-e6>NCOA1, ESR1-e6>CLINT1 and ESR1-e6>TNRC6B demonstrated elevated expression of this gene signature with expression levels comparable to the Y537S and D538G point mutants. Since the LBD point mutants and translocated ESR1 fusions activate a similar pathogenic transcriptional pattern, the gene signature was named "MOTERA" for Mutant or Translocated Estrogen Receptor Alpha. This signature was examined in 20 ER+ patient-derived xenografts and in 55 ER+ MBC samples and successfully identified cases harboring ESR1 fusions.

Gou and colleagues further confirmed the overlap in the transcriptional properties of ESR1-e6>fusions and *ESR1* LBD point mutants in several PDX models and MBC cases. ET-resistant PDXs harboring LBD point mutations (e.g. BCM15100, WHIM20, WHIM40, and HCI013 for ESR1-Y537S; WHIM37 and WHIM43 for ESR1-D538G) highly expressed the MOTERA signature, similar to the PDX naturally expressing the ESR1-e6>YAP1 (WHIM18) fusion. The expression levels of the MOTERA genes were not affected by E2 supplementation in the ESR1-e6>YAP1 expressing PDX or PDXs harboring the LBD point mutations. Furthermore, the MOTERA scores of PDXs expressing WT ESR1 was significantly lower than those of expressing the LBD mutations or the ESR1-e6>YAP1 fusion. Similar to the PDX models, MOTERA gene expression was significantly elevated in MBC tumors harboring the Y537S and D538G point mutations or the ESR1-e6>ARNT2-e18 fusion, and the signature score distinguished the LBD point mutations and the ESR1-e6>ARNT2-e18 fusion from WT *ESR1*.

In addition to the mechanistic studies, evaluating the potential of targeting these fusion proteins for the development of new

targeted therapies is critical. Due to the formation and unique structure of ESR1-e6>fusions, all known ET options that target the LBD are ineffective. Lei and colleagues (80) targeted ER signaling regulated by ESR1 fusions by using Palbociclib, a CDK4/6 inhibitor for MBC. ESR1-e6>YAP1 and ESR1-e6>PCDH11X induced cell proliferation was sensitive to a CDK4/6 inhibition, and a PDX naturally expressing the ESR1-e6>YAP1 fusion was also responsive to Palbociclib treatment. Since ESR1 fusion driven growth of ER-positive breast cancers remained sensitive to CDK4/6 inhibition, the presence of an ESR1-e6>fusion could be a putative biomarker to stratify patients for CDK4/6 inhibitor therapy after resistance to endocrine treatment or continued CDK4/6 inhibitor therapy with a second targeted therapy after resistance to first line treatment for metastatic disease with endocrine therapy in combination with a CDK4/6 inhibitor. It is also hypothesized that CDK4/6 inhibition could be beneficial for patients with ESR1 fusions. Further mechanistic and preclinical studies are expected to introduce additional ESR1 fusions sensitive to CDK4/6 inhibitor therapy and expand on the data from patients harboring ESR1 fusions to examine their CDK4/6 inhibitor responses.

To further explore therapeutic strategies that target ESR1 fusions, Gates and colleagues (99) showed that pharmacological inhibition of ESR1-e6>YAP1 fusion with the proteosome inhibitor MG132, blocked ESR1-e6>YAP1 mediated activation of ER target genes. In the same study, bortezomib, a specific 26S proteasome inhibitor, also suppressed growth driven by the ESR1-e6>YAP1 fusion. Bortezomib was tested in a phase II clinical trial in postmenopausal women with ER+ MBC who had progressive disease after prior aromatase inhibitor therapy. The patients were randomized to fulvestrant and bortezomib versus fulvestrant alone groups. In this study, there was no significant difference in progression free survival, which was the primary end point. However, the combination was overall well tolerated and may have enhanced activity in patients who have an ESR1 fusion (100).

Conclusion and future directions

ET resistance in ER+ breast cancer patients remain a significant clinical problem. The ESR1 fusion proteins are emerging as a mechanism of ET resistance and the studies discussed in this review, deepened our understanding of the prevalence of the ESR1 fusion proteins and the mechanisms by which they drive resistance. The most prevalent and clinically significant ESR1 fusions can be divided into the ESR1-e2>CCDC170 and ESR1-e6>fusion genes. ΔCCDC170, identified in Luminal B breast cancer and generated by ESR1-e2>CCDC170 led to enhanced growth and reduced sensitivity to ET in MBC. ESR1- e6>fusions were identified in ET-resistant MBC and are formed by inter-chromosomal translocation fusing *ESR1* exons 1 to 6 into a 3' fusion partner, replacing the LBD of ESR1. ESR1-e6>fusions drive estradiol-independent growth and constitutive expression of ER target genes leading to ET-resistance.

ESR1 fusions were identified in more aggressive forms of breast cancer (ET resistant MBC and Luminal B breast cancer) and can guide the diagnosis and the development of therapeutic strategies to treat a subset of patients with tumors that harbor these ESR1 alterations. As for the ESR1-e6>fusion genes, only a handful of functionally active ESR1 fusion proteins have been studied to date and therefore ESR1 fusion events remain an understudied form of somatic mutation in breast cancer. The incidence of these ESR1 fusions is still not well understood, but the studies discussed here collectively suggest that the frequency of ESR1 fusions may be higher in heavily pre-treated metastatic samples and when using more sensitive detection techniques.

The discovery of the *ESR1* LBD point mutations has sparked enthusiasm for the development of a new generation of compounds that not only combat existing ER mutants but also inhibit secondary mutations in ER. Indeed, novel oral SERDs and SERCAs are being developed, and likely to be approved in the clinic. Similar to the emergence of *ESR1* LBD mutations that render ligand independent activity, it is likely that the adoption of more potent SERDs and SERCAs will lead to adaptive mechanisms of resistance that are either ER independent or ER dependent but independent of the LBD. It is currently unclear which mechanisms of resistance may emerge following novel SERD and SERCA treatments.

Although, ESR1 gene fusions are rare, the frequency of these fusions may increase under the selective pressure of more effective SERDs and SERCAs. Therefore, better understanding of the mechanism of action of these fusions that lack the LBD, yet drive tumor progression in ER+ MBC, will be critical for the identification of vulnerabilities to target these fusions.

Author contributions

All authors listed have made a substantial contribution to the work and approved it for publication.

Funding

RJ receives NIH/NCI support (5RO1CA237414-02) and support from the Adams Barr Award (DFCI).

Conflict of interest

RJ received research funding from Pfizer and Lilly and is on an advisory board for GE Healthcare.

The remaining author declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Organization WH. *Cancer*. Available at: https://www.who.int/news-room/fact-sheets/detail/cancer.

2. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* (1999) 17 (5):1474–81. doi: 10.1200/JCO.1999.17.5.1474

3. Zwart W, de Leeuw R, Rondaij M, Neefjes J, Mancini MA, Michalides R. The hinge region of the human estrogen receptor determines functional synergy between AF-1 and AF-2 in the quantitative response to estradiol and tamoxifen. *J Cell Sci* (2010) 123(Pt 8):1253–61. doi: 10.1242/jcs.061135

4. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, et al. Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol* (2000) 74(5):311–7. doi: 10.1016/s0960-0760(00)00108-4

5. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, et al. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* (1997) 277(5331):1508–10. doi: 10.1126/science.277.5331.1508

6. Safe S. Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* (2001) 62:231–52. doi: 10.1016/s0083-6729(01)62006-5

7. Safe S, Kim K. Nuclear receptor-mediated transactivation through interaction with sp proteins. *Prog Nucleic Acid Res Mol Biol* (2004) 77:1–36. doi: 10.1016/S0079-6603(04)77001-4

8. Webb P, Nguyen P, Valentine C, Lopez GN, Kwok GR, McInerney E, et al. The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. *Mol Endocrinol* (1999) 13(10):1672–85. doi: 10.1210/mend.13.10.0357

9. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* (1997) 277(5328):965–8. doi: 10.1126/science.277.5328.965

10. Blanco JC, Minucci S, Lu J, Yang XJ, Walker KK, Chen H, et al. The histone acetylase PCAF is a nuclear receptor coactivator. *Genes Dev* (1998) 12(11):1638–51. doi: 10.1101/gad.12.11.1638

11. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, et al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* (1997) 90 (3):569–80. doi: 10.1016/S0092-8674(00)80516-4

12. Hanstein B, Eckner R, DiRenzo J, Halachmi S, Liu H, Searcy B, et al. p300 is a component of an estrogen receptor coactivator complex. *Proc Natl Acad Sci U.S.A.* (1996) 93(21):11540–5. doi: 10.1073/pnas.93.21.11540

13. Hong H, Kohli K, Garabedian MJ, Stallcup MR. GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin d receptors. *Mol Cell Biol* (1997) 17(5):2735–44. doi: 10.1128/MCB.17.5.2735

14. Li H, Gomes PJ, Chen JD. RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc Natl Acad Sci U.S.A.* (1997) 94 (16):8479–84. doi: 10.1073/pnas.94.16.8479

15. Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* (1995) 270 (5240):1354–7. doi: 10.1126/science.270.5240.1354

16. Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, et al. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* (1997) 387(6634):677–84. doi: 10.1038/42652

17. Holmes KA, Hurtado A, Brown GD, Launchbury R, Ross-Innes CS, Hadfield J, et al. Transducin-like enhancer protein 1 mediates estrogen receptor binding and transcriptional activity in breast cancer cells. *Proc Natl Acad Sci U.S.A.* (2012) 109(8):2748–53. doi: 10.1073/pnas.1018863108

18. Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS. FOXA1 is a key determinant of estrogen receptor function and endocrine response. *Nat Genet* (2011) 43(1):27–33. doi: 10.1038/ng.730

19. Kong SL, Li G, Loh SL, Sung WK, Liu ET. Cellular reprogramming by the conjoint action of ERalpha, FOXA1, and GATA3 to a ligand-inducible growth state. *Mol Syst Biol* (2011) 7:526. doi: 10.1038/msb.2011.59

20. Kouros-Mehr H, Kim JW, Bechis SK, Werb Z. GATA-3 and the regulation of the mammary luminal cell fate. *Curr Opin Cell Biol* (2008) 20(2):164–70. doi: 10.1016/j.ceb.2008.02.003

21. Kouros-Mehr H, Slorach EM, Sternlicht MD, Werb Z. GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland. *Cell* (2006) 127 (5):1041–55. doi: 10.1016/j.cell.2006.09.048

22. Lee JH, Bae SB, Oh MH, Cho HD, Jang SH, Hong SA, et al. Clinicopathologic and prognostic significance of transducin-like enhancer of split 1 protein expression in invasive breast cancer. *J Breast Cancer* (2017) 20 (1):45–53. doi: 10.4048/jbc.2017.20.1.45

23. Magnani L, Ballantyne EB, Zhang X, Lupien M. PBX1 genomic pioneer function drives ERalpha signaling underlying progression in breast cancer. *PloS Genet* (2011) 7(11):e1002368. doi: 10.1371/journal.pgen.1002368

24. Patel HK, Bihani T. Selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) in cancer treatment. *Pharmacol Ther* (2018) 186:1–24. doi: 10.1016/j.pharmthera.2017.12.012

25. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med* (2011) 62:233–47. doi: 10.1146/annurev-med-070909-182917

26. Furman C, Hao MH, Prajapati S, Reynolds D, Rimkunas V, Zheng GZ, et al. Estrogen receptor covalent antagonists: The best is yet to come. *Cancer Res* (2019) 79(8):1740–5. doi: 10.1158/0008-5472.CAN-18-3634

27. Puyang X, Furman C, Zheng GZ, Wu ZJ, Banka D, Aithal K, et al. Discovery of selective estrogen receptor covalent antagonists for the treatment of ERalpha (WT) and ERalpha(MUT) breast cancer. *Cancer Discovery* (2018) 8(9):1176–93. doi: 10.1158/2159-8290.CD-17-1229

28. O'Leary B, Finn RS, Turner NC. Treating cancer with selective CDK4/6 inhibitors. *Nat Rev Clin Oncol* (2016) 13(7):417–30. doi: 10.1038/nrclinonc.2016.26

29. Early Breast Cancer Trialists' Collaborative, G. Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials. *Lancet* (2015) 386(10001):1341–52. doi: 10.1016/S0140-6736(15)61074-1

30. Early Breast Cancer Trialists' Collaborative, G. Aromatase inhibitors versus tamoxifen in premenopausal women with oestrogen receptor-positive early-stage breast cancer treated with ovarian suppression: a patient-level meta-analysis of 7030 women from four randomised trials. *Lancet Oncol* (2022) 23(3):382–92. doi: 10.1016/S1470-2045(21)00758-0

31. Aurilio G, Disalvatore D, Pruneri G, Bagnardi V, Viale G, Curigliano G, et al. A meta-analysis of oestrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 discordance between primary breast cancer and metastases. *Eur J Cancer* (2014) 50(2):277–89. doi: 10.1016/j.ejca.2013.10.004

32. Browne AL, Charmsaz S, Vareslija D, Fagan A, Cosgrove N, Cocchiglia S, et al. Network analysis of SRC-1 reveals a novel transcription factor hub which regulates endocrine resistant breast cancer. *Oncogene* (2018) 37(15):2008–21. doi: 10.1038/s41388-017-0042-x

33. Fu X, Jeselsohn R, Pereira R, Hollingsworth EF, Creighton CJ, Li F, et al. FOXA1 overexpression mediates endocrine resistance by altering the ER transcriptome and IL-8 expression in ER-positive breast cancer. *Proc Natl Acad Sci U.S.A.* (2016) 113(43):E6600–9. doi: 10.1073/pnas.1612835113

34. O'Hara J, Vareslija D, McBryan J, Bane F, Tibbitts P, Byrne C, et al. AIB1:ERalpha transcriptional activity is selectively enhanced in aromatase inhibitor-resistant breast cancer cells. *Clin Cancer Res* (2012) 18(12):3305–15. doi: 10.1158/1078-0432.CCR-11-3300

35. Walsh CA, Qin L, Tien JC, Young LS, Xu J. The function of steroid receptor coactivator-1 in normal tissues and cancer. *Int J Biol Sci* (2012) 8(4):470–85. doi: 10.7150/ijbs.4125

36. Zhou J, Xu M, Le K, Ming J, Guo H, Ruan S, et al. SRC promotes tamoxifen resistance in breast cancer *via* up-regulating SIRT1. *Onco Targets Ther* (2020) 13:4635–47. doi: 10.2147/OTT.S245749

37. Zhou Y, Yau C, Gray JW, Chew K, Dairkee SH, Moore DH, et al. Enhanced NF kappa b and AP-1 transcriptional activity associated with antiestrogen resistant breast cancer. *BMC Cancer* (2007) 7:59. doi: 10.1186/1471-2407-7-59

38. Alamolhodaei NS, Behravan J, Mosaffa F, Karimi G. MiR 221/222 as new players in tamoxifen resistance. *Curr Pharm Des* (2016) 22(46):6946–55. doi: 10.2174/1381612822666161102100211

39. Chen MJ, Cheng YM, Chen CC, Chen YC, Shen CJ. MiR-148a and miR-152 reduce tamoxifen resistance in ER+ breast cancer *via* downregulating ALCAM. *Biochem Biophys Res Commun* (2017) 483(2):840–6. doi: 10.1016/j.bbrc.2017.01.012

40. Miller TE, Ghoshal K, Ramaswamy B, Roy S, Datta J, Shapiro CL, et al. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. *J Biol Chem* (2008) 283(44):29897–903. doi: 10.1074/jbc.M804612200

41. Zhang W, Xu J, Shi Y, Sun Q, Zhang Q, Guan X. The novel role of miRNAs for tamoxifen resistance in human breast cancer. *Cell Mol Life Sci* (2015) 72 (13):2575–84. doi: 10.1007/s00018-015-1887-1

42. Chang M. Tamoxifen resistance in breast cancer. *Biomol Ther (Seoul)* (2012) 20(3):256–67. doi: 10.4062/biomolther.2012.20.3.256

43. Christopoulos PF, Msaouel P, Koutsilieris M. The role of the insulin-like growth factor-1 system in breast cancer. *Mol Cancer* (2015) 14:43. doi: 10.1186/s12943-015-0291-7

44. Fan P, Jordan VC. New insights into acquired endocrine resistance of breast cancer. *Cancer Drug Resist* (2019) 2:198–209. doi: 10.20517/cdr.2019.13

45. Turczyk L, Kitowska K, Mieszkowska M, Mieczkowski K, Czaplinska D, Piasecka D, et al. FGFR2-driven signaling counteracts tamoxifen effect on ERalpha-positive breast cancer cells. *Neoplasia* (2017) 19(10):791–804. doi: 10.1016/j.neo.2017.07.006

46. Zhou Y, Wu C, Lu G, Hu Z, Chen Q, Du X. FGF/FGFR signaling pathway involved resistance in various cancer types. *J Cancer* (2020) 11(8):2000–7. doi: 10.7150/jca.40531

47. Abukhdeir AM, Park BH. P21 and p27: roles in carcinogenesis and drug resistance. *Expert Rev Mol Med* (2008) 10:e19. doi: 10.1017/S1462399408000744

48. Abukhdeir AM, Vitolo MI, Argani P, De Marzo AM, Karakas B, Konishi H, et al. Tamoxifen-stimulated growth of breast cancer due to p21 loss. *Proc Natl Acad Sci U.S.A.* (2008) 105(1):288–93. doi: 10.1073/pnas.0710887105

49. Chu I, Sun J, Arnaout A, Kahn H, Hanna W, Narod S, et al. p27 phosphorylation by src regulates inhibition of cyclin e-Cdk2. *Cell* (2007) 128 (2):281–94. doi: 10.1016/j.cell.2006.11.049

50. Jankevicius F, Goebell P, Kushima M, Schulz WA, Ackermann R, Schmitz-Drager BJ. p21 and p53 immunostaining and survival following systemic chemotherapy for urothelial cancer. *Urol Int* (2002) 69(3):174–80. doi: 10.1159/000063949

51. Venditti M, Iwasiow B, Orr FW, Shiu RP. C-myc gene expression alone is sufficient to confer resistance to antiestrogen in human breast cancer cells. *Int J Cancer* (2002) 99(1):35–42. doi: 10.1002/ijc.10269

52. Yu L, Wang L, Mao C, Duraki D, Kim JE, Huang R, et al. Estrogen-independent myc overexpression confers endocrine therapy resistance on breast cancer cells expressing ERalphaY537S and ERalphaD538G mutations. *Cancer Lett* (2019) 442:373–82. doi: 10.1016/j.canlet.2018.10.041

53. Kastrati I, Joosten SEP, Semina SE, Alejo LH, Brovkovych SD, Stender JD, et al. The NF-kappaB pathway promotes tamoxifen tolerance and disease recurrence in estrogen receptor-positive breast cancers. *Mol Cancer Res* (2020) 18(7):1018–27. doi: 10.1158/1541-7786.MCR-19-1082

54. Joffroy CM, Buck MB, Stope MB, Popp SL, Pfizenmaier K, Knabbe C. Antiestrogens induce transforming growth factor beta-mediated immunosuppression in breast cancer. *Cancer Res* (2010) 70(4):1314–22. doi: 10.1158/0008-5472.CAN-09-3292

55. Li D, Ji H, Niu X, Yin L, Wang Y, Gu Y, et al. Tumor-associated macrophages secrete CC-chemokine ligand 2 and induce tamoxifen resistance by activating PI3K/Akt/mTOR in breast cancer. *Cancer Sci* (2020) 111(1):47–58. doi: 10.1111/cas.14230

56. Hermida-Prado F, Jeselsohn R. The ESR1 mutations: From bedside to bench to bedside. *Cancer Res* (2021) 81(3):537–8. doi: 10.1158/0008-5472.CAN-20-4037

57. Jeselsohn R, Bergholz JS, Pun M, Cornwell M, Liu W, Nardone A, et al. Allele-specific chromatin recruitment and therapeutic vulnerabilities of ESR1 activating mutations. *Cancer Cell* (2018) 33(2):173–186 e5. doi: 10.1016/j.ccell.2018.01.004

58. Jeselsohn R, Buchwalter G, De Angelis C, Brown M, Schiff R. ESR1 mutations-a mechanism for acquired endocrine resistance in breast cancer. *Nat Rev Clin Oncol* (2015) 12(10):573–83. doi: 10.1038/nrclinonc.2015.117

59. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R, et al. D538G mutation in estrogen receptor-alpha: A novel mechanism for acquired endocrine resistance in breast cancer. *Cancer Res* (2013) 73(23):6856–64. doi: 10.1158/0008-5472.CAN-13-1197

60. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet* (2013) 45(12):1446–51. doi: 10.1038/ng.2823

61. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet* (2013) 45(12):1439–45. doi: 10.1038/ng.2822

62. Gao Q, Liang WW, Foltz SM, Mutharasu G, Jayasinghe RG, Cao S, et al. Driver fusions and their implications in the development and treatment of human cancers. *Cell Rep* (2018) 23(1):227–238 e3. doi: 10.1016/j.celrep.2018.03.050

63. Dunn DB. Larotrectinib and entrectinib: TRK inhibitors for the treatment of pediatric and adult patients with NTRK gene fusion. *J Adv Pract Oncol* (2020) 11 (4):418–23. doi: 10.6004/jadpro.2020.11.4.9

64. Filippi R, Depetris I, Satolli MA. Evaluating larotrectinib for the treatment of advanced solid tumors harboring an NTRK gene fusion. *Expert Opin Pharmacother* (2021) 22(6):677–84. doi: 10.1080/14656566.2021.1876664

65. Groussin L, Theodon H, Bessiene L, Bricaire L, Bonnet-Serrano F, Cochand-Priollet B, et al. Redifferentiating effect of larotrectinib in NTRK-rearranged advanced radioactive-iodine refractory thyroid cancer. *Thyroid* (2022) 32(5):594–8. doi: 10.1089/thy.2021.0524

66. Hempel D, Wieland T, Solfrank B, Grossmann V, Steinhard J, Frick A, et al. Antitumor activity of larotrectinib in esophageal carcinoma with NTRK gene amplification. *Oncologist* (2020) 25(6):e881–6. doi: 10.1634/theoncologist.2019-0641

67. Laetsch TW, DuBois SG, Mascarenhas L, Turpin B, Federman N, Albert CM, et al. Larotrectinib for paediatric solid tumours harbouring NTRK gene fusions: phase 1 results from a multicentre, open-label, phase 1/2 study. *Lancet Oncol* (2018) 19(5):705–14. doi: 10.1016/S1470-2045(18)30119-0

68. Ricciuti B, Genova C, Crino L, Libra M, Leonardi GC. Antitumor activity of larotrectinib in tumors harboring NTRK gene fusions: a short review on the current evidence. *Onco Targets Ther* (2019) 12:3171–9. doi: 10.2147/OTT.S177051

69. Lei JT, Gou X, Seker S, Ellis MJ. ESR1 alterations and metastasis in estrogen receptor positive breast cancer. *J Cancer Metastasis Treat* (2019) 5. doi: 10.20517/2394-4722.2019.12

70. Loo SK, Yates ME, Yang S, Oesterreich S, Lee AV, Wang XS. Fusion-associated carcinomas of the breast: Diagnostic, prognostic, and therapeutic significance. *Genes Chromosomes Cancer* (2022) 61(5):261–73. doi: 10.1002/gcc.23029

71. Matissek KJ, Onozato ML, Sun S, Zheng Z, Schultz A, Lee J, et al. Expressed gene fusions as frequent drivers of poor outcomes in hormone receptor-positive breast cancer. *Cancer Discovery* (2018) 8(3):336–53. doi: 10.1158/2159-8290.CD-17-0535

72. Veeraraghavan J, Ma J, Hu Y, Wang XS. Recurrent and pathological gene fusions in breast cancer: current advances in genomic discovery and clinical implications. *Breast Cancer Res Treat* (2016) 158(2):219–32. doi: 10.1007/s10549-016-3876-y

73. Jeong JH, Yun JW, Kim HY, Heo CY, Lee S. Elucidation of novel therapeutic targets for breast cancer with ESR1-CCDC170 fusion. *J Clin Med* (2021) 10(4). doi: 10.3390/jcm10040582

74. Li L, Lin L, Veeraraghavan J, Hu Y, Wang X, Lee S, et al. Therapeutic role of recurrent ESR1-CCDC170 gene fusions in breast cancer endocrine resistance. *Breast Cancer Res* (2020) 22(1):84. doi: 10.1186/s13058-020-01325-3

75. Veeraraghavan J, Tan Y, Cao XX, Kim JA, Wang X, Chamness GC, et al. Recurrent ESR1-CCDC170 rearrangements in an aggressive subset of oestrogen receptor-positive breast cancers. *Nat Commun* (2014) 5:4577. doi: 10.1038/ncomms5577

76. Li S, Shen D, Shao J, Crowder R, Liu W, Prat A, et al. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. *Cell Rep* (2013) 4(6):1116–30. doi: 10.1016/j.celrep.2013.08.022

77. Robinson DR, Kalyana-Sundaram S, Wu YM, Shankar S, Cao X, Ateeq B, et al. Functionally recurrent rearrangements of the MAST kinase and notch gene families in breast cancer. *Nat Med* (2011) 17(12):1646–51. doi: 10.1038/nm.2580

78. Tognon C, Knezevich SR, Huntsman D, Roskelley CD, Melnyk N, Mathers JA, et al. Expression of the ETV6-NTRK3 gene fusion as a primary event in human secretory breast carcinoma. *Cancer Cell* (2002) 2(5):367–76. doi: 10.1016/S1535-6108(02)00180-0

79. Vitale SR, Ruigrok-Ritstier K, Timmermans AM, Foekens R, Trapman-Jansen A, Beaufort CM, et al. The prognostic and predictive value of ESR1 fusion gene transcripts in primary breast cancer. *BMC Cancer* (2022) 22(1):165. doi: 10.1186/s12885-022-09265-1

80. Lei JT, Shao J, Zhang J, Iglesia M, Chan DW, Cao J, et al. Functional annotation of ESR1 gene fusions in estrogen receptor-positive breast cancer. *Cell Rep* (2018) 24(6):1434–1444 e7. doi: 10.1016/j.celrep.2018.07.009

81. Gou X, Anurag M, Lei JT, Kim BJ, Singh P, Seker S, et al. Transcriptional reprogramming differentiates active from inactive ESR1 fusions in endocrine therapy-refractory metastatic breast cancer. *Cancer Res* (2021) 81(24):6259–72. doi: 10.1158/0008-5472.CAN-21-1256

82. Giltnane JM, Hutchinson KE, Stricker TP, Formisano L, Young CD, Estrada MV, et al. Genomic profiling of ER(+) breast cancers after short-term estrogen suppression reveals alterations associated with endocrine resistance. *Sci Transl Med* (2017) 9(402):eaai7993. doi: 10.1126/scitranslmed.aai7993

83. Robinson DR, Wu YM, Lonigro RJ, Vats P, Cobain E, Everett J, et al. Integrative clinical genomics of metastatic cancer. *Nature* (2017) 548(7667):297–303. doi: 10.1038/nature23306

84. Hartmaier RJ, Trabucco SE, Priedigkeit N, Chung JH, Parachoniak CA, Vanden Borre P, et al. Recurrent hyperactive ESR1 fusion proteins in endocrine therapy-resistant breast cancer. *Ann Oncol* (2018) 29(4):872–80. doi: 10.1093/annonc/mdy025

85. Priestley P, Baber J, Lolkema MP, Steeghs N, de Bruijn E, Shale C, et al. Pan-cancer whole-genome analyses of metastatic solid tumours. *Nature* (2019) 575 (7781):210–6. doi: 10.1038/s41586-019-1689-y

86. Bruno R, Fontanini G. Next generation sequencing for gene fusion analysis in lung cancer: A literature review. *Diagnostics (Basel)* (2020) 10(8):521. doi: 10.3390/diagnostics10080521

87. Heyer EE, Blackburn J. Sequencing strategies for fusion gene detection. *Bioessays* (2020) 42(7):e2000016. doi: 10.1002/bies.202000016

88. Schroder J, Kumar A, Wong SQ. Overview of fusion detection strategies using next-generation sequencing. *Methods Mol Biol* (2019) 1908:125–38. doi: 10.1007/978-1-4939-9004-7_9

89. Singh RR. Next-generation sequencing in high-sensitive detection of mutations in tumors: Challenges, advances, and applications. *J Mol Diagn* (2020) 22(8):994–1007. doi: 10.1016/j.jmoldx.2020.04.213

90. Supplee JG, Milan MSD, Lim LP, Potts KT, Sholl LM, Oxnard GR, et al. Sensitivity of next-generation sequencing assays detecting oncogenic fusions in plasma cell-free DNA. *Lung Cancer* (2019) 134:96–9. doi: 10.1016/j.lungcan.2019.06.004

91. Chen S, Liu M, Huang T, Liao W, Xu M, Gu J. GeneFuse: detection and visualization of target gene fusions from DNA sequencing data. *Int J Biol Sci* (2018) 14(8):843–8. doi: 10.7150/ijbs.24626

92. Prodduturi N, Bhagwate A, Kocher JA, Sun Z. Indel sensitive and comprehensive variant/mutation detection from RNA sequencing data for precision medicine. *BMC Med Genomics* (2018) 11(Suppl 3):67. doi: 10.1186/s12920-018-0391-5

93. Seager M, Aisner DL, Davies KD. Oncogenic gene fusion detection using anchored multiplex polymerase chain reaction followed by next generation sequencing. *J Vis Exp* (2019) 2019(149):10.3791/59895. doi: 10.3791/59895

94. Teixido C, Gimenez-Capitan A, Molina-Vila MA, Peg V, Karachaliou N, Rodriguez-Capote A, et al. RNA Analysis as a tool to determine clinically relevant gene fusions and splice variants. *Arch Pathol Lab Med* (2018) 142(4):474–9. doi: 10.5858/arpa.2017-0134-RA

95. Ahn SG, Bae SJ, Kim Y, Ji JH, Chu C, Kim D, et al. Primary endocrine resistance of ER+ breast cancer with ESR1 mutations interrogated by droplet digital PCR. *NPJ Breast Cancer* (2022) 8(1):58. doi: 10.1038/s41523-022-00424-y

96. Callens C, Bidard FC, Curto-Taribo A, Trabelsi-Grati O, Melaabi S, Delaloge S, et al. Real-time detection of ESR1 mutation in blood by droplet digital PCR in the PADA-1 trial: Feasibility and cross-validation with NGS. *Anal Chem* (2022) 94(16):6297–303. doi: 10.1021/acs.analchem.2c00446

97. Jeannot E, Darrigues L, Michel M, Stern MH, Pierga JY, Rampanou A, et al. A single droplet digital PCR for ESR1 activating mutations detection in plasma. *Oncogene* (2020) 39(14):2987–95. doi: 10.1038/s41388-020-1174-y

98. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* (2014) 6(224):224ra24. doi: 10.1038/s41388-020-1174-y

99. Gates LA, Gu G, Chen Y, Rohira AD, Lei JT, Hamilton RA, et al. Proteomic profiling identifies key coactivators utilized by mutant ERalpha proteins as potential new therapeutic targets. *Oncogene* (2018) 37(33):4581–98. doi: 10.1038/s41388-018-0284-2

100. Adelson K, Ramaswamy B, Sparano JA, Christos PJ, Wright JJ, Raptis G, et al. Randomized phase II trial of fulvestrant alone or in combination with bortezomib in hormone receptor-positive metastatic breast cancer resistant to aromatase inhibitors: a new York cancer consortium trial. *NPJ Breast Cancer* (2016) 2:16037. doi: 10.1038/npjbcancer.2016.37

frontiers | Frontiers in Oncology

TYPE Correction
PUBLISHED 06 March 2023
DOI 10.3389/fonc.2023.1155540

OPEN ACCESS

EDITED AND APPROVED BY
Ariella Hanker,
University of Texas Southwestern Medical Center, United States

*CORRESPONDENCE
Rinath Jeselsohn
Rinath_Jeselsohn@dfci.harvard.edu
Zsuzsanna Nagy
Zsuzsanna_Nagy@dfci.harvard.edu

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 31 January 2023
ACCEPTED 01 February 2023
PUBLISHED 06 March 2023

CITATION
Nagy Z and Jeselsohn R (2023) Corrigendum: ESR1 fusions and therapeutic resistance in metastatic breast cancer. *Front. Oncol.* 13:1155540. doi: 10.3389/fonc.2023.1155540

Corrigendum: ESR1 fusions and therapeutic resistance in metastatic breast cancer

Zsuzsanna Nagy[1,2,3]* and Rinath Jeselsohn[1,2,3,4]*

[1]Center for Functional Cancer Epigenetics, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, United States, [2]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, United States, [3]Department of Medicine, Harvard Medical School, Boston, MA, United States, [4]Susan F. Smith Center for Women's Cancers, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, United States

KEYWORDS

breast cancer, estrogen receptor, ESR1 fusion, endocrine therapy resistance, SERD

A Corrigendum on
ESR1 fusions and therapeutic resistance in metastatic breast cancer

by Nagy Z and Jeselsohn R (2023) *Front. Oncol.* 12:1037531. doi: 10.3389/fonc.2022.1037531

In the published article, there was an error. In the main text, some ESR1-e6>fusions that Gou and colleagues characterized were incorrectly referred to as transcriptionally inactive.

A correction has been made to "Structure and function of ESR1- e6>fusion proteins in MBC" section, Paragraph 2. This sentence previously stated:

"ESR1-e6>YAP1, ESR1-e6>SOX9, ESR1- e6>ARNT2, ESR1-e6>LPP, and ESR1-e6>NCOA1 produce active fusion proteins that are positive regulators of transcription (80, 81). In contrast to transcriptionally active ESR1-e6>fusions, multiple ESR1-e6>fusions (ESR1-e6>TCF12, ESR1-e6>ARID1B, ESR1- e6>PCDH11X, ESR1-e6>NOP2, ESR1-e6>DAB2, ESR1- e6>CLINT1, ESR1-e6>GRIP1 and ESR1-e6>TNRC6B) were identified as transcriptionally inactive despite producing stable fusion protein, adding to the complex landscape of ESR1- e6>fusion proteins."

The corrected sentence appears below:

"The number of studies investigating the activity of ESR1-e6>fusions is limited, the function of some fusions are still unknown. Further studies are required to investigate and fully validate the stability and activity of ESR1-e6>fusions. Some ESR1-e6>fusions such as ESR1-e6>YAP1, ESR1-e6>SOX9, ESR1- e6>ARNT2, ESR1-e6>LPP, ESR1-e6>NCOA1, ESR1-e6->PCDH11X, ESR1-e6>CLINT1, ESR1-e6>GRIP1 and ESR1-e6>TNRC6B produce stable and active fusion proteins that are positive regulators of transcription (80, 81). ESR1-e6->DAB2 has cell type specific transcriptional activity- active in MCF7 but not T47D cells. In contrast to transcriptionally active ESR1-e6>fusions, multiple ESR1-e6>fusions (e.g. ESR1-e6>TCF12, ESR1-e6>ARID1B, ESR1-e6>NOP2) were identified as transcriptionally inactive despite producing stable fusion protein, adding to the complex landscape of ESR1- e6>fusion proteins."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

Publisher's note

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 19 January 2023
DOI 10.3389/fonc.2022.1105587

OPEN ACCESS

EDITED BY
Svasti Haricharan,
Sanford Burnham Prebys Medical Discovery Institute, United States

REVIEWED BY
Yoshinori Imamura,
Kobe University Hospital, Japan
Anuhya Kommalapati,
Mayo Clinic, United States

*CORRESPONDENCE
Pauline Rottier
p.rottier@baclesse.unicancer.fr

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 22 November 2022
ACCEPTED 22 December 2022
PUBLISHED 19 January 2023

CITATION
Rottier P, Emile G, Johnson A, Levy C, Allouache D, Hrab I, Segura C, Morel A, Villemin M, Dubot-Poitelon C, Boismoreau L, Cherifi F, Lequesne J and Da Silva A (2023) Pretreatment neutrophil to lymphocyte ratio as prognostic factor in metastatic breast cancer treated with cyclin dependent kinase 4/6 inhibitors.
Front. Oncol. 12:1105587.
doi: 10.3389/fonc.2022.1105587

Pretreatment neutrophil to lymphocyte ratio as prognostic factor in metastatic breast cancer treated with cyclin dependent kinase 4/6 inhibitors

Pauline Rottier[1]*, George Emile[1,2], Alison Johnson[1,2], Christelle Levy[1], Djelila Allouache[1], Ioana Hrab[1], Carine Segura[1], Adeline Morel[1], Maud Villemin[1], Coraline Dubot-Poitelon[1], Louis Boismoreau[1], François Cherifi[1], Justine Lequesne[2] and Angélique Da Silva[1]

[1]Breast Cancer Unit, François Baclesse, Comprehensive Cancer Center Institut Normand du Sein, Caen, France, [2]Department of Clinical Research, Francois Baclesse Comprehensive Cancer Center, Caen, France

Background: Cyclin dependent kinase inhibitors (CdK4/6i) changed the course of hormone receptor positive (HR+) HER2 negative (HER2-) metastatic breast cancer (mBC). To date, no factors have been shown to predict response to CdK4/6i. Neutrophil-to-lymphocyte ratio (NLR), an indicator of the host systemic inflammatory response, is an independent prognostic factor for survival in cancers. We conducted this study to evaluate the impact of NLR on survival in mBC patients treated with first line CdK4/6i.

Methods: All mBC patients treated with first line CdK4/6i between November 2015 and December 2019 were retrospectively included. The biomarker threshold was defined using ROC curves. We analyzed progression free survival (PFS), overall survival (OS), 12-month PFS and response rate according to NLR in univariable and multivariable analysis.

Results: A total of 126 patients treated with palbociclib (n=101), ribociclib (n=18) or abemaciclib (n=7) were included, with a median follow-up of 33 months [range: 2.9–57]. Median age was 65 years [29-86], 40% patients had good performance status (ECOG-PS 0). Most patients (71%) were included at the metastatic relapse stage and 29% had only bone metastases. Median PFS and median OS were 27 and 51 months, respectively. High NLR ($\geq$ 2.53) was significantly associated with worse PFS (Hazard Ratio (HR)=0.50, $CI_{95\%}$ = [0.32–0.79]) and worse OS (HR=0.45, [$CI_{95\%}$: 0.23–0.87]). In multivariable analysis, NLR and ECOG PS were independently factors associated with PFS (p=0.016 and p=0.001, respectively).

Conclusion: High NLR was associated with worse PFS and OS in HR+ HER2- mBC patients treated with first line CdK4/6i. NLR is a reliable and inexpensive prognostic marker, easily accessible in routine clinical practice, which could help optimize the therapeutic strategy. These results need to be confirmed in larger prospective studies.

KEYWORDS

metastatic breast cancer, NLR, cyclin dependent kinase inhibitor, prognostic factor, hormone dependent cancer

Introduction

Breast cancer (BC) is the most common malignancy among women and one of the leading causes of death by cancer worldwide (1) despite effective early detection methods and new therapeutic advances. Around 6-10% of BC are diagnosed with *de novo* metastatic disease and 25-30% present a metastatic relapse (2). Metastatic breast cancer (mBC) has a poor survival with a 5-year relative survival rate dropping to around 38% vs. 96% for early BC (eBC), in Europe (3). Approximately 70% of BC are hormone receptor positive (HR+) and human epidermal growth factor receptor 2 negative (HER2-). Endocrine therapy (ET) is the main treatment for patients with HR+/HER2- mBC. The advent of cyclin-dependent kinase inhibitors (CdK4/6i) has considerably improved the prognosis. They are now the gold standard for first line treatment of HR+/HER2- mBC without extensive visceral involvement (3–6).

Prognostic factors are important in estimating outcomes and identifying the optimal treatment for each patient. Some clinical or histological markers are commonly used and validated in HR +/HER2- mBC such as poor Eastern Cooperative Oncology Group Performance Status (ECOG-PS), higher tumor grade and Ki67 expression, negative progesterone receptor (PR) status, prior therapy, sites and number of metastases (multiple vs single), and shorter time to progression to mBC (7). The choice of first-line treatment is crucial, as it affects patients' outcome. However, until now no predictive factor of response to CdK4/6i and ET has been identified. Novel biomarkers are needed to help personalize first line treatment.

Over the last decade, host systemic inflammatory response have been shown to be involved in tumor growth, invasion, angiogenesis and progression (8, 9). This inflammation could be assessed by pretreatment peripheral differential leukocyte count with estimation of lymphocyte count and the calculation of more informative ratios such as neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR) and estimation of lymphocyte count. Several studies in different stages of solid cancers (10–12), including BC, evaluated these ratios and they are now acknowledged as predictive and prognostic factors. In a metanalysis, it was highlighted that high pretreatment NLR was an independent poor prognostic factor for overall survival (OS) and progression-free survival (PFS) in all-stage BC, with the strongest association in the HR+/HER2- subgroup (13). Koh et al. (14) revealed in a prospective study that both NLR and PLR are independently associated with an increased risk of mortality in all-stage BC. However, these inflammation biomarkers have mostly been evaluated in the (neo)adjuvant chemotherapy setting for eBC (15, 16), particularly in triple-negative BC (TNBC) (17). Data remains limited and inconsistent for mBC (18). High LMR before neoadjuvant chemotherapy was reported as a favorable prognostic factor in eBC regardless of HR/HER2 status (19), but no data has been reported for mBC HR+/HER2-.

The aim of our study was to assess the prognostic impact of NLR, lymphopenia, PLR and LMR on survival and response rates in women receiving first line CdK4/6i in association with ET for locally advanced or mBC.

Abbreviations: ALC, Absolute lymphocyte count; BC, Breast Cancer; DFI, Disease-Free Interval; DVT, Deep Vein Thrombosis; ECOG PS, Eastern Cooperative Oncology Group – Performance Status; EMA, European Medicines Agency; ET, Endocrine Therapy; FDA, Food and Drug Administration; HER2-, Human Epidermal growth factor Receptor 2 negative; HR+, Hormone Receptor positive; HR, Hazard ratio; ILD, Interstitial Lung Disease; LMR, Lymphocyte-to-Monocyte Ratio; NLR, Neutrophil-to-Lymphocyte Ratio; NR, Not reached; PE, Pulmonary Embolism; PLR, Platelet-to-Lymphocyte Ratio; TNBC, Triple-Negative Breast Cancer; VTE, Venous ThromboEmbolism.

Methods

Population

We carried out a retrospective single center study at the Comprehensive Cancer Center François Baclesse in Caen, France, as recommended by REMARK (REporting recommendations for

tumor MARKer prognostic studies) for the evaluation of prognostic tumor marker (20). All adult women who received CdK4/6i for histologically proven HR+/HER2- locally advanced or mBC from November 2015 to December 2019 were included. Patients receiving any of the European Medicines Agency (EMA) or Food and Drug Administration (FDA)-approved CdK4/6i (palbociclib, ribociclib, abemaciclib) in association with ET as first-line treatment were included. Patients were excluded if they had received other prior first-line treatment or presented with visceral crisis.

Endpoint

We collected the general characteristics of patients (e.g., age, ECOG-PS, menopausal status), their disease (e.g., TNM staging, hormone receptor expression and SBR (Scarff Bloom Richardson) grade from the primary tumor site or a current metastatic lesion) and prior therapy (adjuvant treatment, palliative radiotherapy or corticosteroid therapy). Results of the blood test performed at the latest the week before starting treatment were collected. NLR was defined as the absolute neutrophil count divided by the absolute lymphocyte count, PLR was defined as the absolute platelet count divided by the absolute lymphocyte count and LMR was defined as the absolute lymphocyte count divided by the absolute monocyte count. Lymphopenia was defined by absolute lymphocyte count (ALC) below 1.5 G/L. Tumor imaging (by computed tomography scan) was performed every 3 cycles and disease response was classified by the radiologist according to the Response Evaluation Criteria in Solid Tumors [RECIST, version 1.1 (21)] as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). Objective response rate (ORR) corresponded to the proportion of patients in whom a CR or PR was observed. Disease control rate (DCR) represented the percentage of patients with either CR, PR, or SD as the best overall response. PFS was defined as the time elapsed between CdK4/6i initiation and radiological progression, death or lost to follow-up. Overall survival (OS) was defined as the time elapsed between CdK4/6i initiation and death from any cause. Adverse events (AE) collected at each medical visit were graded according to National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) version 5.0.

Objectives

The primary objective was to assess the PFS according to pretreatment NLR.

Secondary objectives included assessment of 12-month PFS, OS, ORR and DCR according to pretreatment NLR; assessment of PFS and OS according to lymphopenia, PLR and LMR and evaluation of safety.

Statistical analysis

Descriptive analysis of data provided frequencies and percentages for qualitative variables, and median and extreme values for quantitative variables. Survival curves were estimated by the Kaplan Meier method, and compared by the log-rank test. Multivariable analysis for PFS and OS was performed using Cox's proportional hazards regression model including biological markers significantly associated with survival at a significance level of 0.10 and adjusted on clinical parameters. A stepwise model selection was performed through Akaike's Information Criterion optimization, corresponding to significance-based selection at a significance level of 0.157. The optimal cut-off values for the NLR, PLR and LMR to predict 1-year progression were determined by maximizing the product of sensitivity and specificity, through receiver operating characteristics (ROC) curve analysis. The characteristics of high NLR and low NLR patients were compared by $\chi 2$ test (or Fisher's exact test, in case of observed values per category < 5) for the qualitative variables, and by the Student's t-test for the quantitative variables (or Wilcoxon non-parametric test if data were not normally distributed). Statistical tests and confidence intervals were calculated with an overall risk of 5%. All incident cases were assessed (no calculation of the number of subjects needed). Analyses were conducted using R software, version 4.0.2 (https://cran.r-project.org/bin/windows/base/).

Ethic

The study was in accordance with national regulations regarding research involving human subjects. Registration in the CIL (Correspondant Informatique et Libertés) register was carried out for this study. Patients non-opposition to the use of their data was sought after verification of vital status. All data were anonymized for statistical analysis.

Results

Population

From November 2015 to December 2019, 126 patients were included, with a median follow-up of 33 months (range, 2.9 to 57) (Figure 1: Flow-chart). The median age at inclusion was 65 years (range, 29 to 86). Thirty-six (28.6%) patients presented with *de novo* mBC. 37 patients (29.4%) had bone metastases only, of whom

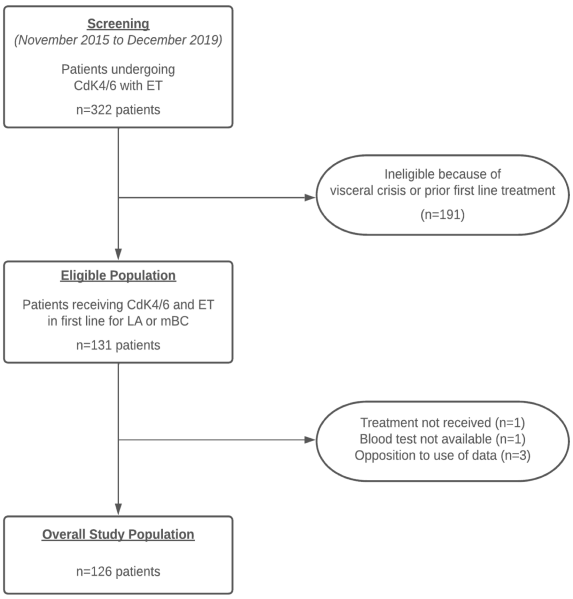


FIGURE 1
Flow-chart.

14 were *de novo* metastatic. The mean disease-free interval (DFI), time between the end of adjuvant treatment before starting any ET and tumor recurrence, was 124.6 months (range, 1 to 360 months). Only 2 patients, one in each NLR group, received chemotherapy in the year preceding the introduction of treatment (last injection 48 days and 51 days before). Thirty patients (23.8%) received radiotherapy within 90 days of beginning ET and CdK4/6i, for a median time interval of 21 days (range, 3 to 66 days). Ten patients had a concomitant prescription of corticosteroid therapy at the first intake of CdK4/6i, with a mean dosage of 35.5 mg. The most commonly prescribed CdK4/6i was palbociclib (n=101, 80%), followed by ribociclib (n=18, 14%) and abemaciclib (n=7, 6%), combined with ET (aromatase inhibitor +/- LHRH analogue for 104 patients (82.5%) or fulvestrant for 22 patients (17.5%)). To the pretreatment stage, median and range of neutrophil, lymphocyte, platelet, and monocyte counts were 3.46 G/L [1.19;14.73], 1.44 G/L [0.14;4.40], 267 G/L [101;622] and 0.49 G/L [0.10;1.30], respectively. Patient characteristics are presented in Table 1.

Overall population outcomes

The median PFS time was 27 months ($CI_{95\%}$= [21–36]), with a 12-month PFS rate of 73.8% ($CI_{95\%}$= [65.7–81.2]). At the end of the follow-up, 61.9% patients (n=78) progressed with first-line metastatic therapy and 31.7% patients (n=40) died. The median OS was 51 months. DCR was 92.1% (16 RC, 64 PR and 36 SD, i.e. 116 patients) and ORR was 63.5% (80 patients).

Prognostic value of NLR

The optimal NLR cut-off value to predict progression within 12 months after metastatic diagnosis was 2.53; 64 patients (50.8%) were classified in the high NLR group (NLR ≥ 2.53). The two groups were similar except for pretreatment ECOG-PS and the occurrence of radiotherapy within 90 days. (Table 2). PFS was significantly better in the low NLR group (Figure 2: PFS and OS probability according to pretreatment NLR) with a median of 39 months compared to the high NLR group with a median of 21.5 months (HR=0.50, [$CI_{95\%}$: 0.32–0.79], log-rank p=0.002). The 12-month PFS rate for the low NLR group was 80.7% [$CI_{95\%}$: 71.4–91.1] versus 65.5% [$CI_{95\%}$: 55.0–78.4] for the high NLR group (Table 3). In a subgroup analysis excluding patients who received radiotherapy within 90 days, we observed the same difference of PFS between the two NLR groups with a HR=0.49 ([$CI_{95\%}$: 0.29–0.83]) in favor of low NLR group.

Low NLR was significantly associated with better OS, HR=0.45 ([$CI_{95\%}$: 0.23–0.87], log-rank p=0.015). Median OS was 43 and 56 months for the high NLR and low NLR group, respectively (Figure 2: PFS and OS probability according to pretreatment NLR).

Distribution of response was significantly different between the low NLR and the high NLR groups (p=0.041), with better response in the low NLR (Table 4). We observed more CR in the low NLR group (n=12, 19.4%) than in the high NLR group (n=4, 6.2%). ORR was 66.2% in the low NLR group and 60.9% in the high NLR group; DCR was 96.8% and 87.5% respectively.

10.3389/fonc.2022.1105587

TABLE 1 Patient characteristics at baseline.

Population characteristics	N = 126 (%)
Median age, years [range]	65 [29;86]
ECOG PS	
0	50 (39.7)
1	62 (49.2)
2	12 (9.5)
3	2 (1.6)
Histology at diagnosis	
Ductal	94 (74.6)
Lobular	29 (23.0)
Other	3 (2.4)
SBR grade at diagnosis	
I	18 (14.3)
II	72 (57.1)
III	32 (25.4)
Unknown	4 (3.0)
Stage at diagnosis	
I	13 (10.8)
II	37 (30.8)
III	34 (27)
IV	36 (28.6)
Unknown	6 (4.8)
Menopause	
Yes	94 (74.6)
No	32 (25.4)
De novo metastatic cancer	
Yes	36 (28.6)
No	90 (71.4)
Metastatic sites	
Locoregional only	3 (2.4)
Bone only	37 (29.4)
Others	86 (68.3)
Adjuvant treatment [a]	
Yes	87 (69)
No	39 (31)
Radiotherapy within 90 days	
Yes	30 (23.8)

(Continued)

TABLE 1 Continued

Population characteristics	N = 126 (%)
No	96 (76.2)
Corticosteroid therapy [b]	
Yes	10 (7.9)
No	116 (92.1)
Blood count (G/L; [range])	
Neutrophils count	3.46 [1.19;14.73]
Lymphocytes count	1.44 [0.14;4.40]
Platelets count	267 [101;622]
Monocytes count	0.49 [0.10;1.30]
NLR (cut-off = 2.53)	
High	64 (51)
Low	62 (49)
Lymphopenia (< 1.5G/L)	
Yes	67 (53)
No	59 (47)
PLR (cut-off = 174.4)	
High	68 (54)
Low	58 (46)
LMR (cut-off = 3.3)	
High	60 (48)
Low	66 (52)

ECOG-PS, Eastern Cooperative Oncology Group – Performance Status; SBR, Scarff-Bloom-Richardson; NLR, Neutrophil to Lymphocyte Ratio; PLR, Platelet to Lymphocyte Ratio; LMR, Lymphocyte to Monocyte Ratio.
[a]including chemiotherapy and/or radiotherapy and/or endocrine therapy.
[b]prior or at baseline.

Prognostic value of lymphopenia and other ratios

Lymphopenia group (n=67 patients) had shorter median PFS, 21 months versus 36 months for patients with normal ALC (HR=0.52, [$CI_{95\%}$: 0.30–0.90], log-rank p=0.068) (Figure 3: PFS and OS probability according to pretreatment ALC). The 12-month PFS rate was 78.0% [$CI_{95\%}$: 68.1–89.3] in the normal ALC group and 68.7% [$CI_{95\%}$: 58.4–80.7] in the lymphopenia group (Table 3). OS was greater in the normal ALC with a 10 months differential on median OS (51 vs 41 months, HR=0.58 [$CI_{95\%}$: 0.30–1.10], log-rank p= 0.09).

The optimal PLR cut-off was 174.4, accounting for 68 patients (54%) in the high PLR group. Pretreatment PLR did not influence PFS (HR=0.73, [$CI_{95\%}$: 0.47–1.15], log-rank p=0.17) with median PFS of 22.6 months in the high PLR group and 36 months in the low PLR group (Table 3). The optimal LMR cut-off was 3.3, accounting for 60 patients (48%) in the high LMR group. Pretreatment LMR did not influence PFS (HR=0.75, [$CI_{95\%}$: 0.48–1.18], log-rank p=0.21), with median PFS of 36 months in the high LMR group and 24.5 months in the low LMR group (Table 3). There was no association between PLR or LMR and OS.

Multivariable analysis

In multivariable analysis, NLR< 2.53 and lymphopenia were included in the model, with adjustment on dose reduction, occurrence of grade 3/4 toxicity, *de novo* metastatic cancer, bone metastases, radiotherapy within 90 days, SBR grade, RP status and ECOG-PS status. Selection model retained NLR< 2.53 and ECOG PS as independently factors associated with PFS, with respectively p=0.016 and p=0.001. NLR< 2.53 was an

TABLE 2 Patient characteristics at baseline according to NLR groups.

Variable	NLR		p value
	High (≥ 2.53) n = 64 (50.8%)	Low (< 2.53) n = 62 (49.2%)	
Median age, years [range]	65 [29;86]	65.5 [32;83]	0.58
ECOG-PS			0.012 [a]
0	18 (28.1)	32 (51.6)	
1, 2 or 3	46 (71.9)	30 (48.4)	
Postmenopausal patients	50 (78.1)	44 (71)	0.47
Prior therapy for eBC [1]	46 (71.9)	41 (66.1)	0.69
De novo stage IV disease	15 (25)	21 (33.9)	0.39
Bone metastases only	19 (29.7)	18 (29)	1
Existence of visceral metastases	38 (59.4)	33 (53.2)	0.61
Radiotherapy within 90 days	23 (35.9)	7 (11.3)	0.0004 [a]
Corticosteroid therapy prior or at treatment initiation	4 (6.3)	6 (9.7)	0.53
iCDK 4/6			0.58
Palbociclib	53 (82.8)	48 (77.4)	
Ribociclib	7 (10.9)	11 (17.8)	
Abemaciclib	4 (6.3)	3 (4.8)	
Endocrinotherapy			0.19
AI	15 (23.4)	7 (11.3)	
Fulvestrant	35 (54.7)	38 (61.3)	
AI + LHRH analog	14 (21.9)	17 (27.4)	

eBC, early Breast Cancer; iCDK 4/6, inhibitor of cyclin dependent kinase 4/6; AI, aromatase inhibitor.
[1]including chemotherapy and/or radiotherapy and/or endocrine therapy.
[a]significant if p<0.05.

independent protector factor with a HR=0.57 ([0.36–0.90]). An impaired of general status (ECOG-PS 1, 2 or 3) was associated with worse survival (HR=2.3 [1.37–3.79]) in multivariable Cox model (Supplementary Table 1).

Safety

The most frequently reported AE were hematologic toxicities with neutropenia (n=110 patients, 87.3%), anemia (n=79, 62.7%) and thrombocytopenia (n=38, 30.2%). Grade 3/4 neutropenia was observed in 57 patients (45%). Only few patients experienced grade 3/4 anemia (n=12, 9.5%) and thrombocytopenia (n=2, 1.6%). Dose reductions were required for 52 patients (41.3%). We also reported 7 venous thromboembolism (VTE) events (n=5.6%), including 2 pulmonary embolism (PE) (n=1.6%) and 5 deep vein thrombosis (DVT) (n=4.0%), and all with Palbociclib which represented 6.9% of patients treated with Palbociclib. None stopped treatment. Two patients (n=1.6%) were suspected of developing interstitial lung disease (ILD), both receiving Palbociclib and with high NLR.

Discussion

Our study highlighted that pretreatment high NLR (≥ 2.53) was a prognostic biomarker associated with worse PFS and OS in women treated with first-line CdK4/6i and ET for metastatic or locally advanced HR+/HER2- breast cancer.

Low NLR appears to be an independent protective factor for PFS and OS with more than 50% risk reduction of progression or death (HR=0.44, [$CI_{95\%}$: 0.23–0.87] for OS). Our results are consistent with previous studies. Four recent meta-analyses corroborate our findings showing that NLR is an independent prognostic factor for PFS and OS in patients with BC at different stages (13, 22, 23), especially for luminal A subtype (24). Wariss et al. (25) reported an association between high NLR and worse

10.3389/fonc.2022.1105587

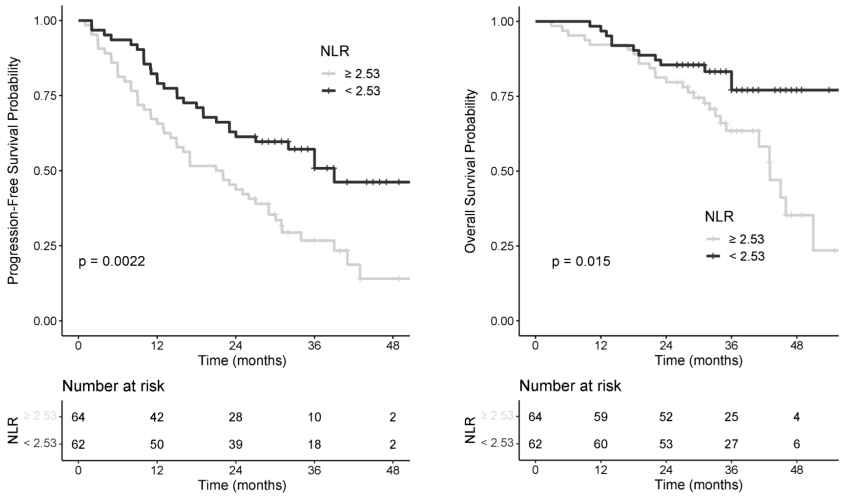


FIGURE 2
PFS and OS probability according to pretreatment NLR.

OS in 2,374 eBC and mBC patients, for patients with luminal subtypes. In another study concerning mTNBC, NLR> 2.5 at diagnosis was a useful predictor of poor OS, regardless of the subsequent treatment (26). In HR+/HER2- eBC, high NLR (>2.25) after neoadjuvant chemotherapy was correlated with poorer disease free survival (DFS) and OS, especially in patients with non-pathologic complete response (pCR) (15). No consensus has been reached to define a cut-off or threshold value for each factor (NLR, lymphopenia, PLR or LMR). We first determined these cut-offs with ROC curves. In our study, NLR cut-off was similar to those found in the literature mostly ranging between 2 and 5. A meta-analysis conducted in BC reported a median NLR cut-off value of 2.5 in 10 out of 15 studies (13). Among different parameters studied, NLR was the only biomarker to show a difference on OS. The median PFS of 27 months in our study was similar to that expected and obtained in the registration trials of CdK4/6 inhibitors (5, 27, 28). To our knowledge, until now our study is the first to report significative prognostic impact of NLR on survival and a benefit in response rates to first line CdK4/6i and ET for HR+/HER2- mBC.

Cell death secondary to breast tumor cells expressing pro-apoptotic ligands and reduced thymic function have been suggested as possible mechanisms of peripheral lymphopenia

TABLE 3 12-month PFS rate according to biomarkers.

Variable	N	Number at risk	12-month PFS rate	$CI_{95\%}$
NLR				
≥ 2.53	64	42	65.6	[55.0 – 78.4]
< 2.53	62	50	80.7	[71.4 – 91.1]
Lymphopenia				
< 1.5 G/L	67	46	68.7	[58.4 – 80.7]
≥ 1.5 G/L	59	46	78.0	[68.1 – 89.3]
PLR				
≥ 174.4	68	47	69.1	[59.0 – 81.0]
< 174.4	58	45	77.6	[67.6 – 89.1]
LMR				
≥ 3.3	60	43	71.7	[61.1 – 84.0]
< 3.3	66	49	74.2	[64.4 – 85.6]

NLR, Neutrophil to Lymphocyte Ratio; PLR, Platelet to Lymphocyte Ratio; LMR, Lymphocyte to Monocyte Ratio.

TABLE 4 Best response according to NLR.

Variable	N	CR (%)	PR (%)	SD (%)	PD (%)	p value
NLR						0.041[a]
≥ 2.53	64	4 (6.2)	35 (54.7)	17 (26.6)	8 (12.5)	
< 2.53	62	12 (19.4)	29 (46.8)	19 (30.6)	2 (3.2)	

NLR, Neutrophil to Lymphocyte Ratio; CR, Complete response; PR, partial Response; SD, Stable disease; PD, Progressive disease.
[a]p< 0.05.

observed in metastatic patients (29). Lymphopenia and NLR are two complementary prognostic factors. Lymphopenia is multifactorial and can be associated with patient characteristics (age, ECOG-PS) (30) or tumor burden and evolves with previous therapies. Increased systemic inflammation markers have been reported in lymphopenic patients, with an inverse increase in the percentage of peripheral neutrophils in response to the expression of pro-inflammatory cytokines such as IL-6 and IL-7, CD4+CD8+ double-positive (DP) thymocytes and an age-related decrease in thymic function or combinatorial T cell receptor diversity (30). The median age was similar between the two groups of pretreatments NLR, but they differed on ECOG-PS: patients in the high NLR group had a worse ECOG-PS. After adjustment on ECOG-PS, NLR was still significantly associated with poorer PFS.

In our study, we observed that more patients with high NLR received radiotherapy within the previous 90 days. This may be explained by the fact that radiotherapy induced lymphopenia can persist for several months (31). In multivariate analysis, we observed that the occurrence of radiotherapy was not associated with PFS. In our study, radiotherapy is not an independent poor prognostic factor and the prolonged lymphopenia may be multifactorial, partly secondary to cancer itself. Systemic treatments (corticosteroid therapy, chemotherapy) could also alter NLR and ALC. We did not observe significant difference regarding corticosteroid therapy between the two NLR groups at baseline. Only one patient in each NLR group received chemotherapy in the months before introduction CdK4/6i but none had presented a disease progression at the time of analysis.

Tumors are infiltrated by leucocytes and produce cytokines and chemokines. Lymphocytes, whether in peripheral blood or as tumor-infiltrating lymphocytes, play a major role in controlling disease progression. In a population of HR+/HER2 mBC patients already treated at least for one metastatic line, we previously showed that those with pretherapeutic ALC< 1.5 G/L had significantly shorter PFS time (6 vs. 10 months, p=0.004), shorter OS time (20 vs. 33 months, p=0.018) and more disease progression at first imaging evaluation (32). The difference on PFS and OS was demonstrated from the onset of lymphopenia. For this reason, we have selected a lymphocyte count of 1.5 G/L to. Although the results are not significant probably due to the lack of power, our study provided further evidence that lymphopenia is a negative prognostic factor for PFS and OS for patients receiving CdK4/6i.

It is necessary to thoroughly understand the impact of the immune system on tumor control. On the one hand, neutrophils, B lymphocytes and some CD4+ T cells may

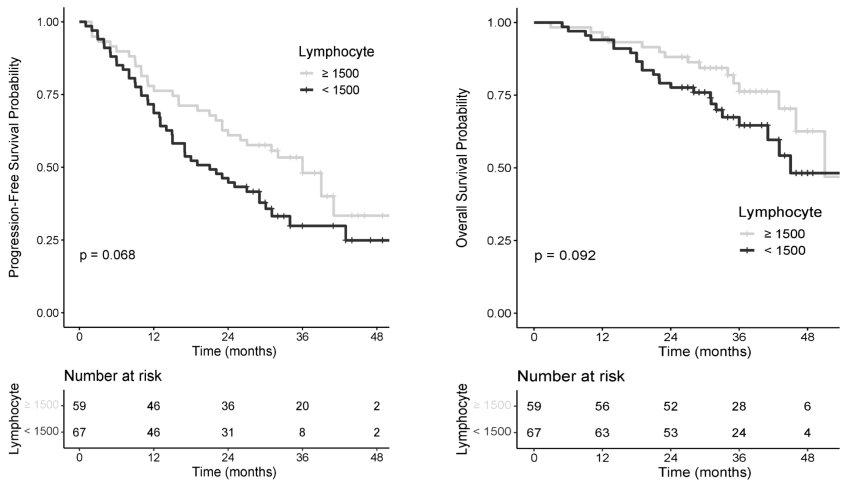


FIGURE 3
PFS and OS probability according to pretreatment ALC.

stimulate cancer growth. On the other hand, cytotoxic CD8+ T cells are crucial components of tumor-specific cellular adaptive immunity as Thelper (TH) 1, TH17, CD4+ T cells and Natural Killer cells are in the tumor microenvironment are. They inhibit tumor growth by producing interferon gamma, subsequently leading to angiostasis, cell cycle inhibition, apoptosis and tumor phagocytosis by macrophages (9). A retrospective study of 1,902 patients with eBC showed that a high total and peripheral CD8+ T cell count was associated with significantly longer breast cancer-specific survival (BCSS) (33). More specifically, in patients with ER-positive tumor, the total number of infiltrating CD8+ T cells was not significantly associated with patient outcome, whereas peripheral CD8+ count was associated with longer BCSS (33). Furthermore, Coffelt et al. demonstrated that elevated neutrophil counts induced by BC tumor cells suppressed CD8+ T cells and promoted metastasis through immunosuppression (34). CdK4/6i have been reported to increase tumor immunogenicity by overcoming two principal mechanisms of tumor immune evasion. They limit the proliferation of regulatory T cells leading to reduced immunosuppression and enhance antitumor immunity by increasing T cell activation, promoting T cell tumor infiltration, and expanding the functional capacity of tumor cells to present antigens (35, 36). This may explain that NLR could be a good biomarker to predict survival and response to CdK4/6i.

Other biomarkers evaluated had no significant impact on survival. PLR has been described as a reliable prognostic marker in many cancers including BC (37, 38). Concerning LMR in the BC neoadjuvant chemotherapy setting, a recent study confirmed this result in a multivariable analysis and showed that patients with low LMR had shorter DFS (16).

Immune status is emerging as an essential biomarker of the tumor biology and microenvironment with an impact on patient outcome. Other biomarkers, such as tumor infiltrating lymphocytes (TILS) and circulating tumor cells (CTC), are still being evaluated in clinical research as prognostic factors but are not easily obtained in routine clinical practice (7).

Despite adjustment on confounding factors, our study had some limitations. Due to the retrospective nature, we were unable to collect the values of some inflammation parameters (albumin, C-reactive protein and LDH). Also we did not have complete information on other discriminating factors of immune response, such as number of B cells, T cells or CD4/CD8 ratio, as these are not routinely performed. The sample size is limited and results must be interpreted with caution. Especially, PLR and LMR were not significant on the primary outcome possibly due to a lack of power, but also because the cut-off determined was not sufficiently discriminating. Moreover, as previously mentioned, there is no NLR threshold recognized in the literature in either breast cancer or any solid cancer, possibly due to its recent identification as a potential prognostic factor. Thus, our 2.53 cut-off NLR obtained by ROC curves requires internal and external validation in future studies.

Our study is the first one concerning the NLR prognostic factor for HR+ HER2- mBC population in first line metastasis only, and treated in this setting with cdk4/6 inhibitors and endocrinotherapy. Our population is therefore notably homogeneous, that increasing the power. Indeed, other studies were interested in the NLR prognostic factor, but their population was inhomogeneous as they included patients at the localized and metastatic stage (39), or metastatic patients only and under cdk4/6 inhibitors but all lines combined without information on previous treatments (40, 41). In this sense, it is an original study.

Nevertheless, in view of these first interesting results, it prompted us to design a prospective study (NCT05303129) in order to complete, confirm and improve these results more powerfully.

Conclusion

Our study highlights NLR as new interesting biomarkers for mBC patients treated with CdK4/6i in the first-line setting. It can be used in routine clinical practice related to it availability, easy-to-use, reliable and inexpensive prognostic factor. These results may allow us to identify different prognostic groups. There are currently few prognostic factors in mBC. To date, none have been validated and are commonly used in first-line metastasis in patients receiving CDK4/6i. Our next project is to validate our results in a prospective study (NCT05303129).

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The datasets generated during and/or analyzed during the current study are not publicly available due to the medically confidential nature of the data but are available from the corresponding author on reasonable request. Requests to access these datasets should be directed to PR. rottier@baclesse.unicancer.fr.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. This is an observational study. The local Research Ethics Committee has confirmed that no ethical approval is required. In accordance with the regulations regarding research involving human subjects, the present study was registered with corresponding data protection. Patients' non-opposition to the use of their data was sought after verification of their vital status.

Author contributions

GE, AS and PR contributed to the study conception and design. Material preparation, data collection and analysis were performed by PR. JL conducted the statistics. The first draft of the manuscript was written by RP and SA, and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1105587/full#supplementary-material

References

1. *The global cancer observatory* (2019).

2. Dawood S, Broglio K, Ensor J, Hortobagyi GN, Giordano SH. Survival differences among women with de novo stage IV and relapsed breast cancer. *Ann Oncol* (2010) 21(11):2169–74. doi: 10.1093/annonc/mdq220

3. Gennari A, André F, Barrios CH, Cortés J, de Azambuja E, DeMichele A, et al. ESMO clinical practice guideline for the diagnosis, staging and treatment of patients with metastatic breast cancer. *Ann Oncol* (2021) 32(12):1475–95. doi: 10.1016/j.annonc.2021.09.019

4. Cardoso F, Senkus E, Costa A, Papadopoulos E, Aapro M, André F, et al. 4th ESO–ESMO international consensus guidelines for advanced breast cancer (ABC 4). *Ann Oncol* (2018) 29(8):1634–57. doi: 10.1093/annonc/mdy192

5. Finn RS, Martin M, Rugo HS, Jones S, Im SA, Gelmon K, et al. Palbociclib and letrozole in advanced breast cancer. *N Engl J Med* (2016) 375(20):1925–36. doi: 10.1056/NEJMoa1607303

6. Cardoso F, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rubio IT, et al. Early breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* (2019) 30(8):1194–220. doi: 10.1016/j.annonc.2021.09.019

7. Cuyún Carter G, Mohanty M, Stenger K, Morato Guimaraes C, Singuru S, Basa P, et al. Prognostic factors in hormone receptor-Positive/Human epidermal growth factor receptor 2-negative (HR+/HER2–) advanced breast cancer: A systematic literature review. *Cancer Manag Res* (2021) 13:6537–66. doi: 10.2147/CMAR.S300869

8. Coussens LM, Werb Z. Inflammation and cancer. *Nature* (2002) 420 (6917):860–7. doi: 10.1038/nature01322

9. Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH. Immune infiltration in human tumors: A prognostic factor that should not be ignored. *Oncogene* (2010) 29(8):1093–102. doi: 10.1038/onc.2009.416

10. Guthrie GJK, Charles KA, Roxburgh CSD, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil–lymphocyte ratio: Experience in patients with cancer. *Crit Rev Oncol Hematol* (2013) 88(1):218–30. doi: 10.1016/j.critrevonc.2013.03.010

11. Clarke SJ, Chua W, Moore M, Kao S, Phan V, Tan C, et al. Use of inflammatory markers to guide cancer treatment. *Clin Pharmacol Ther* (2011) 90 (3):475–8. doi: 10.1038/clpt.2011.122

12. Templeton AJ, McNamara MG, Šeruga B, Vera-Badillo FE, Aneja P, Ocaña A, et al. Prognostic role of neutrophil-to-Lymphocyte ratio in solid tumors: A systematic review and meta-analysis. *JNCI J Natl Cancer Inst* (2014) 106(6): dju124. doi: 10.1093/jnci/dju124

13. Ethier JL, Desautels D, Templeton A, Shah PS, Amir E. Prognostic role of neutrophil-to-lymphocyte ratio in breast cancer: A systematic review and meta-analysis. *Breast Cancer Res* (2017) 19(1):2. doi: 10.1186/s13058-016-0794-1

14. Koh CH, Bhoo-Pathy N, Ng KL, Jabir RS, Tan GH, See MH, et al. Utility of pre-treatment neutrophil–lymphocyte ratio and platelet–lymphocyte ratio as prognostic factors in breast cancer. *Br J Cancer* (2015) 113(1):150–8. doi: 10.1038/bjc.2015.183

15. Koh YW, Lee HJ, Ahn JH, Lee JW, Gong G. Prognostic significance of the ratio of absolute neutrophil to lymphocyte counts for breast cancer patients with ER/PR-positivity and HER2-negativity in neoadjuvant setting. *Tumor Biol* (2014) 35(10):9823–30. doi: 10.1007/s13277-014-2282-5

16. Ma Y, Zhang J, Chen X. Lymphocyte-to-Monocyte ratio is associated with the poor prognosis of breast cancer patients receiving neoadjuvant chemotherapy. *Cancer Manag Res* (2021) 13:1571–80. doi: 10.2147/CMAR.S292048

17. Patel DA, Xi J, Luo J, Hassan B, Thomas S, Ma CX, et al. Neutrophil-to-lymphocyte ratio as a predictor of survival in patients with triple-negative breast cancer. *Breast Cancer Res Treat* (2019) 174(2):443–52. doi: 10.1007/s10549-018-05106-7

18. Ivars Rubio A, Yufera JC, de la Morena P, Fernández Sánchez A, Navarro Manzano E, García Garre E, et al. Neutrophil-lymphocyte ratio in metastatic breast cancer is not an independent predictor of survival, but depends on other variables. *Sci Rep* (2019) 9(1):16979. doi: 10.1038/s41598-019-53606-3

19. Ni XJ, Zhang XL, Ou-Yang QW, Qian GW, Wang L, Chen S, et al. An elevated peripheral blood lymphocyte-to-Monocyte ratio predicts favorable response and prognosis in locally advanced breast cancer following neoadjuvant chemotherapy. *PloS One* (2014) 9(11):e111886. doi: 10.1371/journal.pone.0111886

20. Hayes DF, Ethier S, Lippman ME. New guidelines for reporting of tumor marker studies in breast cancer research and treatment: REMARK. *Breast Cancer Res Treat* (2006) 100(2):237–8. doi: 10.1007/s10549-006-9253-5

21. Schwartz LH, Seymour L, Litière S, Ford R, Gwyther S, Mandrekar S, et al. RECIST 1.1 – standardisation and disease-specific adaptations: Perspectives from the RECIST working group. *Eur J Cancer* (2016) 62:138–45. doi: 10.1016/j.ejca.2016.03.082

22. Wei B, Yao M, Xing C, Wang W, Yao J, Hong Y, et al. The neutrophil lymphocyte ratio is associated with breast cancer prognosis: An updated systematic review and meta-analysis. *OncoTargets Ther* (2016) 9:5567–75. doi: 10.2147/OTT.S108419

23. Liu X, Qu JK, Zhang J, Yan Y, Zhao XX, Wang JZ, et al. Prognostic role of pretreatment neutrophil to lymphocyte ratio in breast cancer patients: A meta-analysis. *Med (Baltimore)* (2017) 96(45):e8101. doi: 10.1097/MD.0000000000008101

24. Noh H, Eomm M, Han A. Usefulness of pretreatment neutrophil to lymphocyte ratio in predicting disease-specific survival in breast cancer patients. *J Breast Cancer.* (2013) 16(1):55. doi: 10.4048/jbc.2013.16.1.55

25. Wariss BR, de Souza Abrahão K, de Aguiar SS, Bergmann A, Thuler LCS. Effectiveness of four inflammatory markers in predicting prognosis in 2374 women with breast cancer. *Maturitas* (2017) 101:51–6. doi: 10.1016/j.maturitas.2017.04.015

26. de la Cruz-Ku G, Chambergo-Michilot D, Torres-Roman JS, Rebaza P, Pinto J, Araujo J, et al. Neutrophil-to-lymphocyte ratio predicts early mortality in

females with metastatic triple-negative breast cancer. *PloS One* (2020) 15(12): e0243447. doi: 10.1371/journal.pone.0243447

27. Hortobagyi GN, Stemmer SM, Burris HA, Yap YS, Sonke GS, Paluch-Shimon S, et al. Updated results from MONALEESA-2, a phase III trial of first-line ribociclib plus letrozole versus placebo plus letrozole in hormone receptor-positive, HER2-negative advanced breast cancer. *Ann Oncol* (2018) 29(7):1541–7. doi: 10.1093/annonc/mdy155

28. Johnston S, Martin M, Di Leo A, Im SA, Awada A, Forrester T, et al. MONARCH 3 final PFS: a randomized study of abemaciclib as initial therapy for advanced breast cancer. *NPJ Breast Cancer* (2019) 5(1):5. doi: 10.1038/s41523-018-0097-z

29. Manuel M, Tredan O, Bachelot T, Clapisson G, Courtier A, Parmentier G, et al. Lymphopenia combined with low TCR diversity (divpenia) predicts poor overall survival in metastatic breast cancer patients. *OncoImmunology* (2012) 1 (4):432–40. doi: 10.4161/onci.19545

30. Ferrando-Martínez S, Franco JM, Hernandez A, Ordoñez A, Gutierrez E, Abad A, et al. Thymopoiesis in elderly human is associated with systemic inflammatory status. *AGE* (2009) 31(2):87–97. doi: 10.1007/s11357-008-9084-x

31. Cesaire M, Le Mauff B, Rambeau A, Toutirais O, Thariat J. Mécanismes de la lymphopénie radio-induite et implications thérapeutiques. *Bull Cancer (Paris)* (2020) 107(7–8):813–22. doi: 10.1016/j.bulcan.2020.04.009

32. Emile G, Penager S, Levy C, Johnson A, Allouache D, Lequesne J, et al. Baseline lymphopenia as prognostic factor in patients with metastatic breast cancer treated with palbociclib. *Oncol Lett* (2021) 23(1):25. doi: 10.3892/ol.2021.13143

33. Mahmoud SMA, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AHS, et al. Tumor-infiltrating CD8 $^{+}$ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol* (2011) 29(15):1949–55. doi: 10.1200/JCO.2010.30.5037

34. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* (2015) 522(7556):345–8. doi: 10.1038/nature14282

35. Goel S, DeCristo MJ, Watt AC, BrinJones H, Sceneay J, Li BB, et al. CDK4/6 inhibition triggers anti-tumour immunity. *Nature* (2017) 548(7668):471–5. doi: 10.1038/nature23465

36. Deng J, Wang ES, Jenkins RW, Li S, Dries R, Yates K, et al. CDK4/6 inhibition augments antitumor immunity by enhancing T-cell activation. *Cancer Discovery* (2018) 8(2):216–33. doi: 10.1158/2159-8290.CD-17-0915

37. Templeton AJ, Ace O, McNamara MG, Al-Mubarak M, Vera-Badillo FE, Hermanns T, et al. Prognostic role of platelet to lymphocyte ratio in solid tumors: A systematic review and meta-analysis. *Cancer Epidemiol Biomarkers Prev* (2014) 23 (7):1204–12. doi: 10.1158/1055-9965.EPI-14-0146

38. Hu Y, Wang S, Ding N, Li N, Huang J, Xiao Z. Platelet/Lymphocyte ratio is superior to Neutrophil/Lymphocyte ratio as a predictor of chemotherapy response and disease-free survival in luminal b-like (HER2–) breast cancer. *Clin Breast Cancer* (2020) 20:e403–9. doi: 10.1016/j.clbc.2020.01.008

39. Wira Wiguna IG, Indrani Remitha NP, Sadvika IGAS, Wiranata S, Putra IW, Adiputra PAT, et al. Pretreatment leukocyte count ratios as metastatic predictive factors in luminal type breast cancer. *Asian Pac J Cancer Prev* (2022) 23(5):1595–601. doi: 10.31557/APJCP.2022.23.5.1595

40. Shikanai A, Horimoto Y, Ishizuka Y, Uomori T, Nakai K, Arakawa A, et al. Clinicopathological features related to the efficacy of CDK4/6 inhibitor-based treatments in metastatic breast cancer. *Breast Cancer Basic Clin Res* (2022) 16:117822342110651. doi: 10.1177/11782234211065148

41. Yilmaz H, Nigdelioglu B, Aytac A, Turan M, Oktay E, Yersal O, et al. The prognostic importance of glucose-to-lymphocyte ratio and uric acid in metastatic breast cancer patients treated with cdk 4/6 inhibitors. *Future Oncol* (2022) 18 (27):3043–53. doi: 10.2217/fon-2022-0464

frontiers | Frontiers in Oncology

TYPE Review
PUBLISHED 25 January 2023
DOI 10.3389/fonc.2023.1111522

OPEN ACCESS

EDITED BY
Maria Rosaria De Miglio,
University of Sassari, Italy

REVIEWED BY
Douglas Faget,
Washington University in St. Louis, United States
Luis Del Valle,
Louisiana State University, United States
Maria Ouzounova,
Institut Curie, France

*CORRESPONDENCE
Heather L. Machado
hmachado@tulane.edu

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 29 November 2022
ACCEPTED 05 January 2023
PUBLISHED 25 January 2023

CITATION
Matherne MG, Phillips ES, Embrey SJ, Burke CM and Machado HL (2023) Emerging functions of C/EBPβ in breast cancer.
Front. Oncol. 13:1111522.
doi: 10.3389/fonc.2023.1111522

Emerging functions of C/EBPβ in breast cancer

Megan G. Matherne[1], Emily S. Phillips[1], Samuel J. Embrey[1], Caitlin M. Burke[1] and Heather L. Machado[1,2]*

[1]Department of Biochemistry and Molecular Biology, Tulane School of Medicine, New Orleans, LA, United States, [2]Tulane Cancer Center, Louisiana Cancer Research Consortium, New Orleans, LA, United States

Breast tumorigenesis relies on complex interactions between tumor cells and their surrounding microenvironment, orchestrated by tightly regulated transcriptional networks. C/EBPβ is a key transcription factor that regulates the proliferation and differentiation of multiple cell types and modulates a variety of biological processes such as tissue homeostasis and the immune response. In addition, C/EBPβ has well-established roles in mammary gland development, is overexpressed in breast cancer, and has tumor-promoting functions. In this review, we discuss context-specific roles of C/EBPβ during breast tumorigenesis, isoform-specific gene regulation, and regulation of the tumor immune response. We present challenges in C/EBPβ biology and discuss the importance of C/EBPβ isoform-specific gene regulation in devising new therapeutic strategies.

KEYWORDS

C/EBPβ, breast cancer, transcription factor, immune cells, gene regulation

1 Introduction

Transcription factors regulate gene expression by recognizing and binding specific DNA sequences such as promoters and enhancers, resulting in either direct or indirect activation or repression of gene transcription. Transcriptional regulation of gene expression is a fundamental biological process that is often modified during cancer initiation, progression, and metastasis (1, 2). Cancer cells adopt mechanisms to escape immune surveillance, evade growth signals, and invade surrounding tissues, and transcription factors are instrumental in driving gene expression programs that aid in acquiring these properties. While much is known about the signaling pathways affecting breast cancer cell growth and apoptosis, fewer studies have addressed transcription factor regulation of gene expression in both the tumor cells and the surrounding microenvironment. CCAAT/enhancer binding protein beta (C/EBPβ), which has long been postulated to promote tumorigenesis and metastasis (3, 4), has more recently emerged as an important transcription factor in both tumor and immune cells.

C/EBPβ is a transcription factor that is one of six members of the C/EBP family, each with highly conserved DNA-binding and basic leucine zipper domains, which form homodimers or heterodimers with one another to bind DNA (5, 6). Three different protein isoforms of C/EBPβ may be translated from the intronless gene: liver-enriched

activating protein (LAP) 1 (also referred to as *LAP), LAP2 (also referred to as LAP), and liver-enriched inhibitory protein (LIP) (5). LAP1 and LAP2 act as transcriptional activators, but LIP lacks the N-terminal transactivation domain while retaining dimerization and instead acts as a dominant negative transcriptional repressor (3, 5). Post-translational modifications are responsible for dictating the transcriptional activity, subcellular localization, and protein-protein interactions of C/EBPβ. C/EBPβ is naturally held in a repressed state by its two regulatory domains that sterically hinder its transactivation domain (6, 7). Sequential phosphorylation at Thr188 followed by Ser184 or Thr179 results in a conformational change where the intramolecular repression of the transactivation domain is relieved, allowing for transcriptional activation. Phosphorylation of C/EBPβ is regulated by many different pathways (8, 9) including Ras-MAPK (10, 11), protein kinases A and C (12), Ca^{2+}/calmodulin dependent protein kinase (13), glycogen synthase kinase 3β (GSK3β) (14), and CDK-cyclin A complexes (15).

C/EBPβ regulates genes involved in proliferation, differentiation, tissue homeostasis, and the immune response in multiple tissue types (4, 8, 16). In the mouse mammary gland, C/EBPβ is required for proper mammary gland development, cellular differentiation, and stem cell activity (17–19). The gene encoding C/EBPβ is generally not mutated in human breast cancers (20). However, increased expression of C/EBPβ is associated with more proliferative and aggressive tumors (21), and a small subset of mammary neoplasms has been shown to have amplification of *Cebpb* (22). Increased C/EBPβ mRNA and protein expression has been associated with triple negative breast cancer (TNBC) (23, 24), although it is unknown whether C/EBPβ expression is altered in other breast cancer subtypes. In syngeneic tumor models, knockdown of C/EBPβ results in decreased tumor latency (25) and tumors with unique histopathologies that include increased inflammation and necrosis (24). The dominant negative isoform LIP is of particular interest, as it has been shown to induce proliferation of mammary epithelial cells (17, 26) and is highly expressed in ER^{neg} tumors with a high proliferative index, compared to ER^{+} breast cancers (4, 26, 27). LIP-deficient transgenic mice ($Cebpb^{\Delta uORF}$) have decreased spontaneous tumor incidence and delayed tumor onset (28–30), whereas mice with elevated LIP (*Wap-LIP* or $Cebpb^{-/L}$) have increased tumor development (4, 31). These studies exemplify the importance of C/EBPβ isoform-specific regulation of gene expression. In this review, we will discuss several mechanisms by which C/EBPβ modulates breast cancer progression, and its implications in therapeutics.

2 Growth-regulatory functions

Sustained proliferative signaling and evasion of growth suppression during cell cycle progression are important processes to ensure the success of tumor progression (32). C/EBPβ has been suggested to mediate these processes through interactions with key cell cycle regulators, including cyclin D1 (33), which is required for cell cycle progression through the G1 phase (34). The cyclin D1 gene (*CCND1*) is frequently amplified in human breast cancer, and similar to C/EBPβ, has a critical role in the differentiation of mammary epithelial cells during pregnancy (35–37). While mammary epithelial cells from $C/EBP\beta^{-/-}$ mice have a block in cell cycle progression at the G1/S transition, cyclin D1 levels remained unchanged, suggesting that C/EBPβ regulation of cell cycle progression is not dependent on cyclin D1 (38). However, cyclin D1 binds to and activates LAP1 to promote mammary epithelial cell differentiation independent of LAP2 and LIP, suggesting a unique interplay between C/EBPβ and cyclin D1 is required for cell differentiation (39). The block in cell cycle progression was associated with decreased cyclin E expression, increased p27 stability, and decreased CDK2 activity (38). The retinoblastoma protein (Rb), a tumor suppressor and G1 checkpoint regulator, has also been shown to directly activate C/EBPβ during cell cycle progression. In addition, C/EBPβ can bind and activate Rb, causing aberrant function of the protein in a pro-tumorigenic fashion (40–43). Furthermore, C/EBPβ has been shown to bind E2F1 and E2F2 to activate E2F targets through CBP/p300, ultimately recruiting chromatin remodeling complexes (44–46). The effect of C/EBPβ binding to either Rb or E2F is not limited to modulating gene transcription but may have an anti-tumorigenic effect by promoting cell cycle exit. In MCF10A cells, the Rb:E2F-dependent senescence pathway requires C/EBPβ for program activation (43). While a direct interaction with Rb:E2F and C/EBPβ has yet to be shown, C/EBPβ acts synergistically with Rb:E2F to repress S-phase associated genes, and C/EBPβ-null cells fail to enter senescence (43, 47).

Additional seemingly paradoxical functions of C/EBPβ are seen in other growth regulation pathways related to transforming growth factor beta (TGFβ). TGFβ normally functions as a growth inhibitor for epithelial cells, acting as a tumor suppressor. In response to TGFβ, LAP2 complexed with FoxO-Smad activates the p15INK4b promoter, while LAP2 complexed with E2F4/5-Smad to repress c-Myc, providing key anti-growth signals. During the switch to metastatic cancer, tumor cells evade growth inhibitory functions of TGFβ by upregulating LIP, causing a block in C/EBPβ-induced p15INK4b activation and relieving c-Myc repression (48). As mentioned previously, TNBC cells have been shown to express a high LIP : LAP ratio, supporting an oncogenic role for LIP (4, 27, 48). These diverse phenotypes demonstrate both anti-tumor and pro-tumor properties of C/EBPβ, which can be attributed to isoform-specific gene regulation (9, 26, 44).

3 Epithelial-mesenchymal transition

In addition to growth-promoting and growth-inhibitory functions, C/EBPβ has been associated with epithelial-mesenchymal transition (EMT), a process where cancer cells acquire mobility and invasive properties due to loss of cell-to-cell junctions (49). TGFβ is a well-known inducer of EMT, and studies have shown that loss of C/EBPβ during the TGFβ response promotes EMT by reducing C/EBPβ-mediated *CDH1* (E-cadherin) transcription (25), suggesting that loss of C/EBPβ is required for EMT. Additionally, in a mouse model used to study the effect of obesity on post-menopausal hormone receptive negative breast cancer, obesity-induced C/EBPβ chromatin binding resulted in elevated expression of MMP9 and claudin-1 (50). In mammary epithelial cells, LIP, but not LAP1/2, is induced by *H-Ras* and was shown to inhibit singleminded 2 (SIM2) gene expression (51). SIM2 is a tumor suppressor that is downregulated in mammary epithelial cells at periods where LIP is

the dominant C/EBPβ isoform. SIM2 represses MMP3 and SLUG (52), both of which induce EMT (53, 54). Knockdown of SIM2 is associated with decreased E-cadherin and increased MMP2, N-cadherin and vimentin (55). These reports suggest that LIP may indirectly induce EMT by repressing SIM2. A recent study shows that LIP promotes cell migration in untransformed MCF10A cells and LAP expression reduces migration in TNBC cell lines (BT-20 and BT-549) (56). In contrast, overexpression of LAP2 in MCF10A cells *in vitro* has been shown to induce EMT-like morphologies, accompanied by delocalized E-cadherin and increased vimentin (57). Several studies have shown that LAP2 binds to and activates cyclooxygenase-2 (COX-2) gene expression (58–65), which promotes TGFβ-induced EMT in a PGE_2-dependent manner (66). Together, these reports suggest C/EBPβ isoform-specific roles in regulating EMT, although further studies are required to define these mechanisms.

4 Tumor-promoting inflammation

4.1 Tumor-derived C/EBPβ

Tumor-promoting inflammation is another hallmark of cancer, where cancer cells adopt inflammatory mechanisms to promote their growth and survival (32). C/EBPβ, initially identified as nuclear factor for interleukin-6 expression (NF-IL6) (67, 68), has important roles in mediating the inflammatory response. Early studies indicated that C/EBPβ binds to an IL-1 response element in the IL-6 promoter to drive IL-6 and IL-8 transcription. C/EBPβ and other C/EBP family members can directly interact with the Rel homology domain between NF-κB subunits p50, p65 and c-Rel, stabilizing NF-κB, leading to synergistic transcriptional activation of IL-6 and IL-8 (69, 70). C/EBPβ can also positively regulate NF-κB by binding and inactivating IκBα, the canonical inhibitor of NF-κB (71). In breast cancer cells, C/EBPβ can act in synergy with STAT3 to activate c-Jun activation domain-binding protein (Jab)-1, which regulates cycle control, apoptosis, and DNA repair (72). More recently, STAT3 was shown to stabilize C/EBPβ in *H-Ras* transformed mammary epithelial cells, to cooperatively induce the transcription of cancer-promoting inflammatory cytokines (70, 73). C/EBPβ was also shown to directly bind to the STAT5 promoter, facilitating TNBC cell growth and invasion *in vitro* (23). These results demonstrate that with other key transcription factors, C/EBPβ coordinately promotes the induction of various inflammatory cytokines during tumor progression.

Chemokine signaling facilitates leukocyte recruitment and activation and can dictate the balance between the pro- and anti-tumor immune response. In the tumor microenvironment, tumor and immune cells, such as tumor-infiltrating macrophages, secrete chemokines to promote immune evasion, growth and survival of tumor cells, angiogenesis, and metastasis (74). *Cebpb*$^{-/-}$ mice have defective helper T cell function and lymphoproliferative diseases (75), which are in part due to alterations in cytokine and chemokine production. Knockdown of C/EBPβ in mouse mammary tumor 4T1 cells results in increased expression of various chemokines such as in CCL6, CCL7, CCL8, CCL12, CCL27, CCL28, and CXCL16 expression, although whether C/EBPβ directly inhibits these chemokines was not addressed (24). Other studies have shown that tumor-derived C/EBPβ directly activates various chemokines, including CCL2, CCL5, CXCL12 and CXCR4 (76–80). In contrast, LIP was shown to bind to and inhibit the CCL2 promoter (78), and LIP can indirectly activate CXCR4 by inhibiting the CXCR4 repressor YYI (80). These opposing roles for LIP in chemokine activation/inhibition may be due to microenvironment- and tumor context-specificity, however, further studies are required to address these differences. C/EBPβ has also been shown to cooperate with ATF to activate RANKL (81). Notably, while a number of studies have shown altered chemokine expression in cancer cells after C/EBPβ knockdown, few studies have validated whether and how LAP/LIP directly bind to chemokine promoters.

In addition to recruiting tumor-promoting immune cells, breast cancer cells evade cytotoxic effector cells by downregulating the expression of receptor-bound recognition proteins. Transformed cells actively downregulate immunogenic surface receptors to avoid immune recognition and destruction by cytotoxic $CD8^+$ T cells (82–84). A recent study showed that deletion of C/EBPβ in human breast cancer cells (BT-20) results in the de-repression of genes involved in immune visibility, where MHC I and MHC II gene sets were significantly upregulated as compared to wildtype (56). In another study, C/EBPβ-silenced 4T1 cells were shown to have significantly upregulated expression of MHCIIα, MHCIIβ and HLACIIγ (24). While functional antigen presentation assays were not performed, C/EBPβ-silenced 4T1 tumors displayed a significant increase in $CD3^+$ lymphocytes *in vivo* (24). Finally, C/EBPβ was shown to repress type I and type II interferon response genes in a mouse model of *Ras*-induced squamous papilloma (85). These studies suggest that C/EBPβ may aide in immune escape, although further investigation is required to define the specific mechanisms.

4.2 Myeloid-derived C/EBPβ

While studies have focused on how tumor-derived C/EBPβ inhibits or promotes tumorigenesis, C/EBPβ is also expressed in immune cells, and in particular has important roles in myeloid cells. C/EBPβ has well-established roles in myelopoiesis (86, 87), and overexpression of LAP1 or deletion of C/EBPβ in THP-1 cells causes decreased monocyte proliferation (88). More recently, C/EBPβ was shown to be required for $Ly6C^+$ monocyte differentiation into $Ly6C^-$ cells, through a mechanism involving C/EBPβ activation of *Nr4a1* (89). In addition, *Cebpb*$^{-/-}$ bone marrow-derived macrophages have impaired phagocytic function (75, 90). Macrophage-derived C/EBPβ also modulates the balance between pro- and anti-inflammatory signals during tissue repair. In the wound healing process, pro-inflammatory cytokines such as IL-6, TNFα and IL-1β induce C/EBPβ activation in macrophages, which in turn activates these cytokines in a feedback loop to modulate inflammation (87, 91). While transcription factor regulation of macrophages has been studied in response to injury, less is known about how macrophage C/EBPβ regulates tumor progression. In a syngeneic mouse model of early-stage breast cancer progression, *Cebpb* was highly expressed in numerous macrophage populations identified by single cell RNA sequencing, as well as in *Ly6c2*$^+$ monocytes, and *S100a8*$^+$ neutrophils, the latter of which likely give rise to MDSCs (92). In metastatic melanoma, macrophages with high C/EBPβ expression

10.3389/fonc.2023.1111522

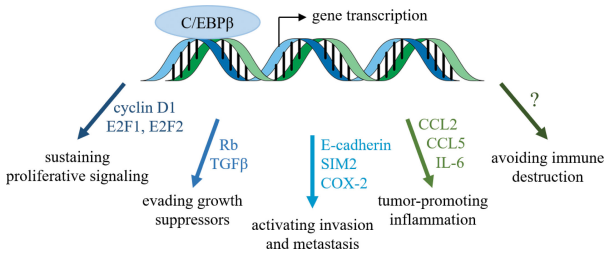


FIGURE 1
Transcription factor C/EBPβ regulates several hallmarks of cancer. C/EBPβ influences the cell cycle and epithelial-mesenchymal transition of breast cancer cells, as well as the tumor microenvironment.

had significantly higher activation of the IL6-JAK-STAT3 signaling pathway (93). These data suggest that C/EBPβ may have crucial roles in regulating the functions of tumor-infiltrating macrophages, although further investigation is required to validate this idea.

C/EBPβ has been shown to be a key transcription factor regulator of MDSCs. C/EBPβ is required for the differentiation and expansion of MDSCs in the bone marrow of septic mice, by inducing miR-21a, miR-21b and miR-181b in a STAT3-dependent manner (94, 95). In tumor models, genetic ablation of C/EBPβ from MDSCs isolated from tumors impairs T-cell suppressor activity (96). C/EBPβ can induce arginase I expression in response to injury (97, 98), providing one potential mechanism for C/EBPβ-mediated immune-suppressive function of MDSCs during cancer progression. In colon cancer cells, Gao et al. identified a long noncoding RNA, termed lnc-C/EBPβ, which binds to LIP to inhibit the activation of C/EBPβ target genes, such as *Arg1*, *Nos2*, *Nox2 and Cox2*, and thus negatively regulates immune-suppressive functions of MDSCs (99). In TNBC, LAP2 was shown to promote the recruitment of MDSCs by activating G-CSF and GM-CSF through a tumor-specific glycolysis-dependent pathway (100, 101). Together, these studies suggest that both tumor- and myeloid-derived C/EBPβ are important regulators of MDSC immune-suppressive function.

TABLE 1 Isoform-specific regulation of gene expression and protein function.

Isoform	Gene or protein	Result	References
LAP1	Binds cyclin D1	Mammary epithelial cell differentiation	(33, 38, 39)
LAP2	Binds FoxO-Smad complex	Tumor suppressor p15INK4b promoter is activated	(48)
	Binds E2F4/5-Smad complex	Proto-oncogene c-Myc promoter is repressed	(48)
	Delocalized expression of E-cadherin and increased expression of vimentin	EMT phenotype is expressed	(57)
	Increased expression of E-cadherin and decreased expression of vimentin	Epithelial phenotype is maintained	(25)
	Activates COX-2 expression	EMT phenotype is expressed	(58–66)
LIP	Inhibits LAP2 from binding FoxO-Smad complex	Blocks transcription of tumor suppressor p15INK4b	(48)
	Inhibits LAP2 from binding E2F4/5-Smad complex	Proto-oncogene c-Myc is expressed	(48)
	Inhibits SIM2	EMT phenotype is expressed	(51)
	Inhibits CCL2 expression	Reduces pro-inflammatory phenotype	(78, 113)
	Binds and inhibits YY1	CXCR4 expression is activated	(80)
Isoform unknown or not specified	Binds Rb	Activates C/EBPβ, activates Rb	(40, 42)
	Binds E2F1 and E2F2	Recruits chromatin remodeling complexes	(45, 46)
	Interacts with Rb:E2F	Induces cell senescence	(43, 47)
	Regulates Rb/E2F/cyclin E pathway	Reduces monocyte proliferation	(88)
	Binds NF-κB	IL-6 and IL-8 expression is activated	(67–69, 76, 100, 114, 115)
	Binds IκBα	NF-κB is not inhibited	(71)
	Interacts with STAT3	Stabilizes C/EBPβ, activates Jab-1	(70, 72, 73)
	Activates STAT5 expression	JAK/STAT pathway is activated	(23)
	Activates CCL2 and CCL5 expression	Promotes metastasis	(76–78, 116–119)
	Regulates CXCL12 expression	CXCR4/CXCL12 axis promotes metastasis	(79, 80)
	Inhibits MHCI and MHCII	Reduced antigen recognition	(24, 56)
	Activates arginase I expression	Macrophages take on anti-inflammatory phenotype	(97, 98)

5 Discussion

C/EBPβ has emerged as a critical transcription factor for successful breast tumor progression, promoting cancer cell growth and survival, metastasis, inflammation, and potentially immune evasion (Figure 1). C/EBPβ has also been implicated in therapeutic resistance. In colorectal cancer, FOXO1/C/EBPβ/NF-κB signaling is required for CCL20-dependent recruitment of regulatory T cells, which confer chemoresistance to 5-fluorouracil (102). Radiation resistance of nasopharyngeal carcinoma has been attributed to the PGC1α/C/EBPβ/CPT1A axis (103), and C/EBPβ is required for therapeutic resistance in NRF2-activated non-small cell lung cancer (104). Thus, C/EBPβ may be an attractive target in overcoming therapeutic resistance.

While transcription factors have traditionally been considered "undruggable," emerging studies have focused on overcoming the challenges associated with targeting transcription factors as a therapeutic strategy (105, 106). A recent study demonstrated the ability of a selective peptide C/EBPβ antagonist, ST101, to induce ubiquitin-dependent C/EBPβ degradation, resulting in tumor growth inhibition in xenograft models (107). Similarly, cell-penetrating peptides Bpep and Dpep have been designed to act as leucine zipper decoys with specificity for tumor C/EBPβ (108). Other studies have shown that LIP can be inhibited by pharmacological inhibition of mTORC1, suggesting that rapamycin analogues may be an effective therapeutic strategy (29, 109). Synthetic analogues of helenalin covalently bind and inhibit C/EBPβ and have been shown to reduce proliferation in acute myeloid leukemia cells *in vitro* (110, 111). In non-small cell lung cancer, metformin reduced tumor growth *via* the AMPK/C/EBPβ/PD-L1 axis (112). The efficacy of these small molecule inhibitors in breast cancer has yet to be determined.

Despite emerging studies demonstrating the importance of C/EBPβ in breast cancer progression, much remains to be learned about the gene regulatory networks induced by C/EBPβ during tumor progression. The complexity of C/EBPβ isoform-specific gene regulation (Table 1) has largely hindered our understanding of C/EBPβ-induced gene expression in both tumor and immune cells. LAP1/LAP2 and LIP can have opposing functions within the same pathway or program, yet many valuable studies lack details on which isoform of C/EBPβ regulates gene expression and protein function. The lack of isoform-specific antibodies continues to present a significant challenge for both basic science and clinical studies (44). For example, defining isoform specificity at different stages of breast cancer progression may provide insights on whether isoform expression, and downstream targets, are of prognostic value. Despite these limitations, advances in genetic mouse models and gene editing technology has recently allowed for the study of individual isoforms, by using approaches that delete C/EBPβ while simultaneously overexpressing LAP2 or LIP (31). Future studies should focus on understanding isoform-specific functions in both the tumor and immune cells, which will have critical implications for developing therapeutic strategies that target either specific C/EBPβ isoforms, such as LIP, or C/EBPβ-induced target pathways.

Author contributions

MM: Manuscript writing, conception of the topic. EP: Manuscript writing. SE: Manuscript writing. CB: Manuscript editing and revision. HM: Conception of the topic, manuscript editing and revision, funding. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by NIH R01CA212518 and R01HD106929 to HM.

Acknowledgments

We thank Dr. James Jackson (Tulane University) for helpful discussions and comments regarding these topics.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Nebert DW. Transcription factors and cancer: An overview. *Toxicology* (2002) 181-182:131–41. doi: 10.1016/S0300-483X(02)00269-X

2. Islam Z, Ali AM, Naik A, Eldaw M, Decock J, Kolatkar PR. Transcription factors: The fulcrum between cell development and carcinogenesis. *Front Oncol* (2021) 11:681377. doi: 10.3389/fonc.2021.681377

3. Raught B, Gingras AC, James A, Medina D, Sonenberg N, Rosen JM. Expression of a translationally regulated, dominant-negative Ccaat/Enhancer-binding protein beta isoform and up-regulation of the eukaryotic translation initiation factor 2alpha are correlated with neoplastic transformation of mammary epithelial cells. *Cancer Res* (1996) 56(19):4382–6.

4. Zahnow CA, Younes P, Laucirica R, Rosen JM. Overexpression of C/Ebpbeta-lip, a naturally occurring, dominant-negative transcription factor, in human breast cancer. *J Natl Cancer Inst* (1997) 89(24):1887–91. doi: 10.1093/jnci/89.24.1887

5. Descombes P, Schibler U. A liver-enriched transcriptional activator protein, lap, and a transcriptional inhibitory protein, lip, are translated from the same mrna. *Cell* (1991) 67 (3):569–79. doi: 10.1016/0092-8674(91)90531-3

6. Kowenz-Leutz E, Twamley G, Ansieau S, Leutz A. Novel mechanism of C/Ebp beta (Nf-m) transcriptional control: Activation through derepression. *Genes Dev* (1994) 8 (22):2781–91. doi: 10.1101/gad.8.22.2781

7. Williams SC, Baer M, Dillner AJ, Johnson PF. Crp2 (C/Ebp beta) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. *EMBO J* (1995) 14(13):3170–83. doi: 10.1002/j.1460-2075.1995.tb07319.x

8. Takiguchi M. The C/Ebp family of transcription factors in the liver and other organs. *Int J Exp Pathol* (1998) 79(6):369–91. doi: 10.1046/j.1365-2613.1998.00082.x

9. Zahnow CA. Ccaat/Enhancer-binding protein beta: Its role in breast cancer and associations with receptor tyrosine kinases. *Expert Rev Mol Med* (2009) 11:e12. doi: 10.1017/s1462399409001033

10. Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T, et al. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor nf-Il6. *Proc Natl Acad Sci U.S.A.* (1993) 90 (6):2207–11. doi: 10.1073/pnas.90.6.2207

11. Mo X, Kowenz-Leutz E, Xu H, Leutz A. Ras induces mediator complex exchange on C/Ebp beta. *Mol Cell* (2004) 13(2):241–50. doi: 10.1016/s1097-2765(03)00521-5

12. Trautwein C, van der Geer P, Karin M, Hunter T, Chojkier M. Protein kinase a and c site-specific phosphorylations of lap (Nf-Il6) modulate its binding affinity to DNA recognition elements. *J Clin Invest* (1994) 93(6):2554–61. doi: 10.1172/jci117266

13. Wegner M, Cao Z, Rosenfeld MG. Calcium-regulated phosphorylation within the leucine zipper of C/Ebp beta. *Science* (1992) 256(5055):370–3. doi: 10.1126/science.256.5055.370

14. Tang QQ, Grønborg M, Huang H, Kim JW, Otto TC, Pandey A, et al. Sequential phosphorylation of ccaat enhancer-binding protein beta by mapk and glycogen synthase kinase 3beta is required for adipogenesis. *Proc Natl Acad Sci U.S.A.* (2005) 102(28):9766–71. doi: 10.1073/pnas.0503891102

15. Shuman JD, Sebastian T, Kaldis P, Copeland TD, Zhu S, Smart RC, et al. Cell cycle-dependent phosphorylation of C/Ebpbeta mediates oncogenic cooperativity between C/Ebpbeta and h-Rasv12. *Mol Cell Biol* (2004) 24(17):7380–91. doi: 10.1128/mcb.24.17.7380-7391.2004

16. Lekstrom-Himes J, Xanthopoulos KG. Biological role of the Ccaat/Enhancer-binding protein family of transcription factors. *J Biol Chem* (1998) 273(44):28545–8. doi: 10.1074/jbc.273.44.28545

17. Robinson GW, Johnson PF, Hennighausen L, Sterneck E. The C/Ebpbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes Dev* (1998) 12(12):1907–16. doi: 10.1101/gad.12.12.1907

18. Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, et al. C/Ebpbeta, but not C/Ebpalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. *Genes Dev* (1998) 12(12):1917–28. doi: 10.1101/gad.12.12.1917

19. LaMarca HL, Visbal AP, Creighton CJ, Liu H, Zhang Y, Behbod F, et al. Ccaat/Enhancer binding protein beta regulates stem cell activity and specifies luminal cell fate in the mammary gland. *Stem Cells* (2010) 28(3):535–44. doi: 10.1002/stem.297

20. Vegesna V, Takeuchi S, Hofmann WK, Ikezoe T, Tavor S, Krug U, et al. C/Ebp-beta, C/Ebp-delta, Pu.1, Aml1 genes: Mutational analysis in 381 samples of hematopoietic and solid malignancies. *Leuk Res* (2002) 26(5):451–7. doi: 10.1016/s0145-2126(01)00150-3

21. Zahnow CA. Ccaat/Enhancer binding proteins in normal mammary development and breast cancer. *Breast Cancer Res* (2002) 4(3):113. doi: 10.1186/bcr428

22. Mastracci TL, Shadeo A, Colby SM, Tuck AB, O'Malley FP, Bull SB, et al. Genomic alterations in lobular neoplasia: A microarray comparative genomic hybridization signature for early neoplastic proliferationin the breast. *Genes Chromosomes Cancer* (2006) 45(11):1007–17. doi: 10.1002/gcc.20368

23. Wang S, Xia D, Wang X, Cao H, Wu C, Sun Z, et al. C/Ebpβ regulates the Jak/Stat signaling pathway in triple-negative breast cancer. *FEBS Open Bio* (2021) 11(4):1250–8. doi: 10.1002/2211-5463.13138

24. Kurzejamska E, Johansson J, Jirström K, Prakash V, Ananthaseshan S, Boon L, et al. C/Ebpβ expression is an independent predictor of overall survival in breast cancer patients by Mhcii/Cd4-dependent mechanism of metastasis formation. *Oncogenesis* (2014) 3(11):e125. doi: 10.1038/oncsis.2014.38

25. Johansson J, Berg T, Kurzejamska E, Pang MF, Tabor V, Jansson M, et al. Mir-155-Mediated loss of C/Ebpβ shifts the tgf-B response from growth inhibition to epithelial-mesenchymal transition, invasion and metastasis in breast cancer. *Oncogene* (2013) 32 (50):5614–24. doi: 10.1038/onc.2013.322

26. Zahnow CA, Cardiff RD, Laucirica R, Medina D, Rosen JM. A role for Ccaat/Enhancer binding protein beta-Liver-Enriched inhibitory protein in mammary epithelial cell proliferation. *Cancer Res* (2001) 61(1):261–9.

27. Milde-Langosch K, Löning T, Bamberger AM. Expression of the Ccaat/Enhancer-binding proteins C/Ebpalpha, C/Ebpbeta and C/Ebpdelta in breast cancer: Correlations with clinicopathologic parameters and cell-cycle regulatory proteins. *Breast Cancer Res Treat* (2003) 79(2):175–85. doi: 10.1023/a:1023929504884

28. Wethmar K, Bégay V, Smink JJ, Zaragoza K, Wiesenthal V, Dörken B, et al. C/Ebpbetadeltauorf mice–a genetic model for uorf-mediated translational control in mammals. *Genes Dev* (2010) 24(1):15–20. doi: 10.1101/gad.557910

29. Zidek LM, Ackermann T, Hartleben G, Eichwald S, Kortman G, Kiehntopf M, et al. Deficiency in Mtorc1-controlled C/Ebpβ-mrna translation improves metabolic health in mice. *EMBO Rep* (2015) 16(8):1022–36. doi: 10.15252/embr.201439837

30. Müller C, Zidek LM, Ackermann T, de Jong T, Liu P, Kliche V, et al. Reduced expression of C/Ebpβ-lip extends health and lifespan in mice. *Elife* (2018) 7. doi: 10.7554/eLife.34985

31. Bégay V, Smink JJ, Loddenkemper C, Zimmermann K, Rudolph C, Scheller M, et al. Deregulation of the endogenous C/Ebpβ lip isoform predisposes to tumorigenesis. *J Mol Med (Berl)* (2015) 93(1):39–49. doi: 10.1007/s00109-014-1215-5

32. Hanahan D. Hallmarks of cancer: New dimensions. *Cancer Discovery* (2022) 12 (1):31–46. doi: 10.1158/2159-8290.Cd-21-1059

33. Lamb J, Ramaswamy S, Ford HL, Contreras B, Martinez RV, Kittrell FS, et al. A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. *Cell* (2003) 114(3):323–34. doi: 10.1016/s0092-8674(03)00570-1

34. Sherr CJ. Cancer cell cycles. *Science* (1996) 274(5293):1672–7. doi: 10.1126/science.274.5293.1672

35. Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, et al. Expression and amplification of cyclin genes in human breast cancer. *Oncogene* (1993) 8 (8):2127–33.

36. Bartkova J, Lukas J, Müller H, Lützhøft D, Strauss M, Bartek J. Cyclin D1 protein expression and function in human breast cancer. *Int J Cancer* (1994) 57(3):353–61. doi: 10.1002/ijc.2910570311

37. Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, et al. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* (1995) 82(4):621–30. doi: 10.1016/0092-8674(95)90034-9

38. Grimm SL, Contreras A, Barcellos-Hoff MH, Rosen JM. Cell cycle defects contribute to a block in hormone-induced mammary gland proliferation in Ccaat/Enhancer-binding protein (C/Ebpbeta)-null mice. *J Biol Chem* (2005) 280(43):36301–9. doi: 10.1074/jbc.M508167200

39. Liu Q, Boudot A, Ni J, Hennessey T, Beauparlant SL, Rajabi HN, et al. Cyclin D1 and C/Ebpβ Lap1 operate in a common pathway to promote mammary epithelial cell differentiation. *Mol Cell Biol* (2014) 34(16):3168–79. doi: 10.1128/mcb.00039-14

40. Chen PL, Riley DJ, Chen-Kiang S, Lee WH. Retinoblastoma protein directly interacts with and activates the transcription factor nf-Il6. *Proc Natl Acad Sci U.S.A.* (1996) 93(1):465–9. doi: 10.1073/pnas.93.1.465

41. Chen PL, Riley DJ, Chen Y, Lee WH. Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/Ebps. *Genes Dev* (1996) 10(21):2794–804. doi: 10.1101/gad.10.21.2794

42. Charles A, Tang X, Crouch E, Brody JS, Xiao ZX. Retinoblastoma protein complexes with C/Ebp proteins and activates C/Ebp-mediated transcription. *J Cell Biochem* (2001) 83(3):414–25. doi: 10.1002/jcb.1239

43. Sebastian T, Malik R, Thomas S, Sage J, Johnson PF. C/Ebpbeta cooperates with Rb:E2f to implement Ras(V12)-induced cellular senescence. *EMBO J* (2005) 24(18):3301–12. doi: 10.1038/sj.emboj.7600789

44. Spike AJ, Rosen JM. C/Ebpß isoform specific gene regulation: It's a lot more complicated than you think! *J Mammary Gland Biol Neoplasia* (2020) 25(1):1–12. doi: 10.1007/s10911-020-09444-5

45. Wang H, Larris B, Peiris TH, Zhang L, Le Lay J, Gao Y, et al. C/Ebpbeta activates E2f-regulated genes in vivo *Via* recruitment of the coactivator creb-binding Protein/P300. *J Biol Chem* (2007) 282(34):24679–88. doi: 10.1074/jbc.M705066200

46. Mink S, Haenig B, Klempnauer KH. Interaction and functional collaboration of P300 and C/Ebpbeta. *Mol Cell Biol* (1997) 17(11):6609–17. doi: 10.1128/mcb.17.11.6609

47. Sebastian T, Johnson PF. Stop and go: Anti-proliferative and mitogenic functions of the transcription factor C/Ebpbeta. *Cell Cycle* (2006) 5(9):953–7. doi: 10.4161/cc.5.9.2733

48. Gomis RR, Alarcón C, Nadal C, Van Poznak C, Massagué J. C/Ebpbeta at the core of the tgfbeta cytostatic response and its evasion in metastatic breast cancer cells. *Cancer Cell* (2006) 10(3):203–14. doi: 10.1016/j.ccr.2006.07.019

49. Nieto MA. Epithelial plasticity: A common theme in embryonic and cancer cells. *Science* (2013) 342(6159):1234850. doi: 10.1126/science.1234850

50. Liu X-Z, Rulina A, Choi MH, Pedersen L, Lepland J, Takle ST, et al. C/Ebpb-dependent adaptation to palmitic acid promotes tumor formation in hormone receptor negative breast cancer. *Nat Commun* (2022) 13(1):69. doi: 10.1038/s41467-021-27734-2

51. Gustafson TL, Wellberg E, Laffin B, Schilling L, Metz RP, Zahnow CA, et al. Ha-ras transformation of Mcf10a cells leads to repression of singleminded-2s through notch and C/Ebpbeta. *Oncogene* (2009) 28(12):1561–8. doi: 10.1038/onc.2008.497

52. Kwak HI, Gustafson T, Metz RP, Laffin B, Schedin P, Porter WW. Inhibition of breast cancer growth and invasion by single-minded 2s. *Carcinogenesis* (2007) 28(2):259–66. doi: 10.1093/carcin/bgl122

53. Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, et al. The stromal proteinase Mmp3/Stromelysin-1 promotes mammary carcinogenesis. *Cell* (1999) 98(2):137–46. doi: 10.1016/s0092-8674(00)81009-0

54. Cichon MA, Nelson CM, Radisky DC. Regulation of epithelial-mesenchymal transition in breast cancer cells by cell contact and adhesion. *Cancer Inform* (2015) 14 (Suppl 3):1–13. doi: 10.4137/cin.S18965

55. Laffin B, Wellberg E, Kwak H-I, Burghardt RC, Metz RP, Gustafson T, et al. Loss of singleminded-2s in the mouse mammary gland induces an epithelial-mesenchymal transition associated with up-regulation of slug and matrix metalloprotease 2. *Mol Cell Biol* (2008) 28(6):1936–46. doi: 10.1128/mcb.01701-07

56. Sterken BA, Ackermann T, Müller C, Zuidhof HR, Kortman G, Hernandez-Segura A, et al. C/Ebpβ isoform-specific regulation of migration and invasion in triple-negative breast cancer cells. *NPJ Breast Cancer* (2022) 8(1):11. doi: 10.1038/s41523-021-00372-z

57. Bundy LM, Sealy L. Ccaat/Enhancer binding protein beta (C/Ebpbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture. *Oncogene* (2003) 22(6):869–83. doi: 10.1038/sj.onc.1206216

58. Kim Y, Fischer SM. Transcriptional regulation of cyclooxygenase-2 in mouse skin carcinoma cells. regulatory role of Ccaat/Enhancer-binding proteins in the differential expression of cyclooxygenase-2 in normal and neoplastic tissues. *J Biol Chem* (1998) 273 (42):27686–94. doi: 10.1074/jbc.273.42.27686

59. Reddy ST, Wadleigh DJ, Herschman HR. Transcriptional regulation of the cyclooxygenase-2 gene in activated mast cells. *J Biol Chem* (2000) 275(5):3107–13. doi: 10.1074/jbc.275.5.3107

60. Thomas B, Berenbaum F, Humbert L, Bian H, Béréziat G, Crofford L, et al. Critical role of C/Ebpdelta and C/Ebpbeta factors in the stimulation of the cyclooxygenase-2 gene transcription by interleukin-1beta in articular chondrocytes. *Eur J Biochem* (2000) 267 (23):6798–809. doi: 10.1046/j.1432-1033.2000.01778.x

61. Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated raw 264.7 macrophages. *J Biol Chem* (2000) 275(9):6259–66. doi: 10.1074/jbc.275.9.6259

62. Yuan CJ, Mandal AK, Zhang Z, Mukherjee AB. Transcriptional regulation of cyclooxygenase-2 gene expression: Novel effects of nonsteroidal anti-inflammatory drugs. *Cancer Res* (2000) 60(4):1084–91.

63. Caivano M, Gorgoni B, Cohen P, Poli V. The induction of cyclooxygenase-2 mrna in macrophages is biphasic and requires both ccaat enhancer-binding protein beta (C/Ebp beta) and C/Ebp delta transcription factors. *J Biol Chem* (2001) 276(52):48693–701. doi: 10.1074/jbc.M108282200

64. Gorgoni B, Caivano M, Arizmendi C, Poli V. The transcription factor C/Ebpbeta is essential for inducible expression of the cox-2 gene in macrophages but not in fibroblasts. *J Biol Chem* (2001) 276(44):40769–77. doi: 10.1074/jbc.M106865200

65. Wardlaw SA, Zhang N, Belinsky SA. Transcriptional regulation of basal cyclooxygenase-2 expression in murine lung tumor-derived cell lines by Ccaat/Enhancer-binding protein and activating transcription Factor/Camp response element-binding protein. *Mol Pharmacol* (2002) 62(2):326–33. doi: 10.1124/mol.62.2.326

66. Neil JR, Johnson KM, Nemenoff RA, Schiemann WP. Cox-2 inactivates smad signaling and enhances emt stimulated by tgf-beta through a Pge2-dependent mechanisms. *Carcinogenesis* (2008) 29(11):2227–35. doi: 10.1093/carcin/bgn202

67. Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, et al. A nuclear factor for il-6 expression (Nf-Il6) is a member of a C/Ebp family. *EMBO J* (1990) 9(6):1897–906. doi: 10.1002/j.1460-2075.1990.tb08316.x

68. Poli V, Mancini FP, Cortese R. Il-6dbp, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/Ebp. *Cell* (1990) 63(3):643–53. doi: 10.1016/0092-8674(90)90459-r

69. Stein B, Cogswell PC, Baldwin ASJr. Functional and physical associations between nf-kappa b and C/Ebp family members: A rel domain-bzip interaction. *Mol Cell Biol* (1993) 13(7):3964–74. doi: 10.1128/mcb.13.7.3964-3974.1993

70. Lee LL, Kim SJ, Hahn YI, Jang JH, Saeidi S, Surh YJ. Stabilization of C/Ebpβ through direct interaction with Stat3 in h-ras transformed human mammary epithelial cells. *Biochem Biophys Res Commun* (2021) 546:130–7. doi: 10.1016/j.bbrc.2021.02.011

71. Cappello C, Zwergal A, Kanclerski S, Haas SC, Kandemir JD, Huber R, et al. C/Ebpβ enhances nf–Kb-Associated signalling by reducing the level of iκb-A. *Cell signal* (2009) 21(12):1918–24. doi: 10.1016/j.cellsig.2009.08.009

72. Shackleford TJ, Zhang Q, Tian L, Vu TT, Korapati AL, Baumgartner AM, et al. Stat3 and Ccaat/Enhancer binding protein beta (C/Ebp-beta) regulate Jab1/Csn5 expression in mammary carcinoma cells. *Breast Cancer Res* (2011) 13(3):R65. doi: 10.1186/bcr2902

73. McClure C, McPeak MB, Youssef D, Yao ZQ, McCall CE, El Gazzar M. Stat3 and C/Ebpβ synergize to induce mir-21 and mir-181b expression during sepsis. *Immunol Cell Biol* (2017) 95(1):42–55. doi: 10.1038/icb.2016.63

74. Chow MT, Luster AD. Chemokines in cancer. *Cancer Immunol Res* (2014) 2 (12):1125–31. doi: 10.1158/2326-6066.Cir-14-0160

75. Screpanti I, Romani L, Musiani P, Modesti A, Fattori E, Lazzaro D, et al. Lymphoproliferative disorder and imbalanced T-helper response in C/Ebp beta-deficient mice. *EMBO J* (1995) 14(9):1932–41. doi: 10.1002/j.1460-2075.1995.tb07185.x

76. Bretz JD, Williams SC, Baer M, Johnson PF, Schwartz RC. C/Ebp-related protein 2 confers lipopolysaccharide-inducible expression of interleukin 6 and monocyte chemoattractant protein 1 to a lymphoblastic cell line. *Proc Natl Acad Sci U.S.A.* (1994) 91(15):7306–10. doi: 10.1073/pnas.91.15.7306

77. Wickremasinghe MI, Thomas LH, O'Kane CM, Uddin J, Friedland JS. Transcriptional mechanisms regulating alveolar epithelial cell-specific Ccl5 secretion in pulmonary tuberculosis. *J Biol Chem* (2004) 279(26):27199–210. doi: 10.1074/jbc.M403107200

78. Spooner CJ, Guo X, Johnson PF, Schwartz RC. Differential roles of C/Ebp beta regulatory domains in specifying mcp-1 and il-6 transcription. *Mol Immunol* (2007) 44 (6):1384–92. doi: 10.1016/j.molimm.2006.05.004

79. Calonge E, Alonso-Lobo JM, Escandón C, González N, Bermejo M, Santiago B, et al. C/Ebpbeta is a major regulatory element driving transcriptional activation of the Cxcl12 promoter. *J Mol Biol* (2010) 396(3):463–72. doi: 10.1016/j.jmb.2009.11.064

80. Park BH, Kook S, Lee S, Jeong JH, Brufsky A, Lee BC. An isoform of C/Ebpβ, lip, regulates expression of the chemokine receptor Cxcr4 and modulates breast cancer cell migration. *J Biol Chem* (2013) 288(40):28656–67. doi: 10.1074/jbc.M113.509505

81. Tsushima H, Okazaki K, Ishihara K, Ushijima T, Iwamoto Y. Ccaat/Enhancer-binding protein B promotes receptor activator of nuclear factor-Kappa-B ligand (Rankl) expression and osteoclast formation in the synovium in rheumatoid arthritis. *Arthritis Res Ther* (2015) 17(1):31. doi: 10.1186/s13075-015-0532-6

82. Lollini PL, Nicoletti G, Landuzzi L, De Giovanni C, Rossi I, Di Carlo E, et al. Down regulation of major histocompatibility complex class I expression in mammary carcinoma of her-2/Neu transgenic mice. *Int J Cancer* (1998) 77(6):937–41. doi: 10.1002/(sici)1097-0215(19980911)77:6<937::aid-ijc24>3.0.co;2-x

83. Herrmann F, Lehr HA, Drexler I, Sutter G, Hengstler J, Wollscheid U, et al. Her-2/Neu-Mediated regulation of components of the mhc class I antigen-processing pathway. *Cancer Res* (2004) 64(1):215–20. doi: 10.1158/0008-5472.can-2522-2

84. Inoue M, Mimura K, Izawa S, Shiraishi K, Inoue A, Shiba S, et al. Expression of mhc class I on breast cancer cells correlates inversely with Her2 expression. *Oncoimmunology* (2012) 1(7):1104–10. doi: 10.4161/onci.21056

85. Messenger ZJ, Hall JR, Jima DD, House JS, Tam HW, Tokarz DA, et al. C/Ebpβ deletion in oncogenic ras skin tumors is a synthetic lethal event. *Cell Death Dis* (2018) 9 (11):1054. doi: 10.1038/s41419-018-1103-y

86. Tamura A, Hirai H, Yokota A, Kamio N, Sato A, Shoji T, et al. C/Ebpβ is required for survival of Ly6c(-) monocytes. *Blood* (2017) 130(16):1809–18. doi: 10.1182/blood-2017-03-772962

87. Huber R, Pietsch D, Panterodt T, Brand K. Regulation of C/Ebpβ and resulting functions in cells of the monocytic lineage. *Cell Signal* (2012) 24(6):1287–96. doi: 10.1016/j.cellsig.2012.02.007

88. Gutsch R, Kandemir JD, Pietsch D, Cappello C, Meyer J, Simanowski K, et al. Ccaat/Enhancer-binding protein beta inhibits proliferation in monocytic cells by affecting the retinoblastoma Protein/E2f/Cyclin e pathway but is not directly required for macrophage morphology. *J Biol Chem* (2011) 286(26):22716–29. doi: 10.1074/jbc.M110.152538

89. Mildner A, Schönheit J, Giladi A, David E, Lara-Astiaso D, Lorenzo-Vivas E, et al. Genomic characterization of murine monocytes reveals C/Ebpβ transcription factor dependence of Ly6c(-) cells. *Immunity* (2017) 46(5):849–62.e7. doi: 10.1016/j.immuni.2017.04.018

90. Tanaka T, Akira S, Yoshida K, Umemoto M, Yoneda Y, Shirafuji N, et al. Targeted disruption of the nf-Il6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell* (1995) 80(2):353–61. doi: 10.1016/0092-8674(95)90418-2

91. Tengku-Muhammad TS, Hughes TR, Ranki H, Cryer A, Ramji DP. Differential regulation of macrophage ccaat-enhancer binding protein isoforms by lipopolysaccharide and cytokines. *Cytokine* (2000) 12(9):1430–6. doi: 10.1006/cyto.2000.0711

92. Ibrahim AM, Moss MA, Gray Z, Rojo MD, Burke CM, Schwertfeger KL, et al. Diverse macrophage populations contribute to the inflammatory microenvironment in premalignant lesions during localized invasion. *Front Oncol* (2020) 10:569985(1812). doi: 10.3389/fonc.2020.569985

93. Yang J, Xu Y, Xie K, Gao L, Zhong W, Liu X. Cebpb is associated with active tumor immune environment and favorable prognosis of metastatic skin cutaneous melanoma. *Front Immunol* (2022) 13:991797. doi: 10.3389/fimmu.2022.991797

94. Dai J, Kumbhare A, Youssef D, Yao ZQ, McCall CE, El Gazzar M. Expression of C/Ebpβ in myeloid progenitors during sepsis promotes immunosuppression. *Mol Immunol* (2017) 91:165–72. doi: 10.1016/j.molimm.2017.09.008

95. Dai J, Kumbhare A, Williams DA, Youssef D, Yao ZQ, McCall CE, et al. Nfia deletion in myeloid cells blocks expansion of myeloid-derived suppressor cells during sepsis. *Innate Immun* (2018) 24(1):54–65. doi: 10.1177/1753425917742956

96. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, et al. Tumor-induced tolerance and immune suppression depend on the C/Ebpbeta transcription factor. *Immunity* (2010) 32(6):790–802. doi: 10.1016/j.immuni.2010.05.010

97. Ruffell D, Mourkioti F, Gambardella A, Kirstetter P, Lopez RG, Rosenthal N, et al. A creb-C/Ebpβ cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci* (2009) 106(41):17475–80. doi: 10.1073/pnas.0908641106

98. Nishikoba N, Kumagai K, Kanmura S, Nakamura Y, Ono M, Eguchi H, et al. Hgf-met signaling shifts M1 macrophages toward an M2-like phenotype through Pi3k-mediated induction of arginase-1 expression. *Front Immunol* (2020) 11:2135. doi: 10.3389/fimmu.2020.02135

99. Gao Y, Sun W, Shang W, Li Y, Zhang D, Wang T, et al. Lnc-C/Ebpβ negatively regulates the suppressive function of myeloid-derived suppressor cells. *Cancer Immunol Res* (2018) 6(11):1352–63. doi: 10.1158/2326-6066.Cir-18-0108

100. Dunn SM, Coles LS, Lang RK, Gerondakis S, Vadas MA, Shannon MF. Requirement for nuclear factor (Nf)-kappa b P65 and nf-Interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. *Blood* (1994) 83(9):2469–79. doi: 10.1182/blood.V83.9.2469.2469

101. Li W, Tanikawa T, Kryczek I, Xia H, Li G, Wu K, et al. Aerobic glycolysis controls myeloid-derived suppressor cells and tumor immunity *Via* a specific cebpb isoform in triple-negative breast cancer. *Cell Metab* (2018) 28(1):87–103.e6. doi: 10.1016/j.cmet.2018.04.022

102. Wang D, Yang L, Yu W, Wu Q, Lian J, Li F, et al. Colorectal cancer cell-derived Ccl20 recruits regulatory T cells to promote chemoresistance *Via* Foxo1/Cebpb/Nf-Kb signaling. *J Immunother Cancer* (2019) 7(1):215. doi: 10.1186/s40425-019-0701-2

103. Du Q, Tan Z, Shi F, Tang M, Xie L, Zhao L, et al. Pgc1α/Cebpb/Cpt1a axis promotes radiation resistance of nasopharyngeal carcinoma through activating fatty acid oxidation. *Cancer Sci* (2019) 110(6):2050–62. doi: 10.1111/cas.14011

104. Okazaki K, Anzawa H, Katsuoka F, Kinoshita K, Sekine H, Motohashi H. Cebpb is required for Nrf2-mediated drug resistance in Nrf2-activated non-small cell lung cancer cells. *J Biochem* (2022) 171(5):567–78. doi: 10.1093/jb/mvac013

105. Bushweller JH. Targeting transcription factors in cancer — from undruggable to reality. *Nat Rev Cancer* (2019) 19(11):611–24. doi: 10.1038/s41568-019-0196-7

106. Henley MJ, Koehler AN. Advances in targeting 'Undruggable' transcription factors with small molecules. *Nat Rev Drug Discovery* (2021) 20(9):669–88. doi: 10.1038/s41573-021-00199-0

107. Darvishi E, Ghamsari L, Leong SF, Ramirez R, Koester M, Gallagher E, et al. Anticancer activity of St101, a novel antagonist of Ccaat/Enhancer binding protein B. *Mol Cancer Ther* (2022) 21(11):1632–44. doi: 10.1158/1535-7163.Mct-21-0962

108. Zhou Q, Sun X, Pasquier N, Jefferson P, Nguyen TTT, Siegelin MD, et al. Cell-penetrating cebpb and cebpd leucine zipper decoys as broadly acting anti-cancer agents. *Cancers* (2021) 13(10):2504. doi: 10.3390/cancers13102504

109. Jundt F, Raetzel N, Müller C, Calkhoven CF, Kley K, Mathas S, et al. A rapamycin derivative (Everolimus) controls proliferation through down-regulation of truncated ccaat enhancer binding protein {Beta} and nf-{Kappa}B activity in Hodgkin and anaplastic Large cell lymphomas. *Blood* (2005) 106(5):1801–7. doi: 10.1182/blood-2004-11-4513

110. Abdel Ghani L, Yusenko MV, Frank D, Moorthy R, Widen JC, Dörner W, et al. A synthetic covalent ligand of the C/Ebpβ transactivation domain inhibits acute myeloid leukemia cells. *Cancer Lett* (2022) 530:170–80. doi: 10.1016/j.canlet.2022.01.024

111. Jakobs A, Steinmann S, Henrich SM, Schmidt TJ, Klempnauer K-H. Helenalin acetate, a natural sesquiterpene lactone with anti-inflammatory and anti-cancer activity, disrupts the cooperation of ccaat Box/Enhancer-binding protein β (C/Ebpβ); and Co-activator P300 *. *J Biol Chem* (2016) 291(50):26098–108. doi: 10.1074/jbc.M116.748129

112. Lu T, Li M, Zhao M, Huang Y, Bi G, Liang J, et al. Metformin inhibits human non-small cell lung cancer by regulating ampk–Cebpb–Pdl1 signaling pathway. *Cancer Immunol Immunother* (2022) 71(7):1733–46. doi: 10.1007/s00262-021-03116-x

113. Fei L, Ren X, Yu H, Zhan Y. Targeting the Ccl2/Ccr2 axis in cancer immunotherapy: One stone, three birds? *Front Immunol* (2021) 12:771210. doi: 10.3389/fimmu.2021.771210

114. Stein B, Baldwin ASJr. Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/Ebp and nf-kappa b. *Mol Cell Biol* (1993) 13(11):7191–8. doi: 10.1128/mcb.13.11.7191-7198.1993

115. Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, et al. Transcription factors nf-Il6 and nf-kappa b synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc Natl Acad Sci U.S.A.* (1993) 90(21):10193–7. doi: 10.1073/pnas.90.21.10193

116. Yoshimura T, Howard OM, Ito T, Kuwabara M, Matsukawa A, Chen K, et al. Monocyte chemoattractant protein-1/Ccl2 produced by stromal cells promotes lung metastasis of 4t1 murine breast cancer cells. *PloS One* (2013) 8(3):e58791. doi: 10.1371/journal.pone.0058791

117. Velasco-Velázquez M, Pestell RG. The Ccl5/Ccr5 axis promotes metastasis in basal breast cancer. *Oncoimmunology* (2013) 2(4):e23660. doi: 10.4161/onci.23660

118. Araujo JM, Gomez AC, Aguilar A, Salgado R, Balko JM, Bravo L, et al. Effect of Ccl5 expression in the recruitment of immune cells in triple negative breast cancer. *Sci Rep* (2018) 8(1):4899. doi: 10.1038/s41598-018-23099-7

119. Walens A, DiMarco AV, Lupo R, Kroger BR, Damrauer JS, Alvarez JV. Ccl5 promotes breast cancer recurrence through macrophage recruitment in residual tumors. *Elife* (2019) 8. doi: 10.7554/eLife.43653

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 14 March 2023
DOI 10.3389/fonc.2023.1075298

OPEN ACCESS

EDITED BY
Martin Rakuša,
Maribor University Medical Centre, Slovenia

REVIEWED BY
Masoud Sadeghi,
Islamic Azad University, Iran
Maja Ravnik,
Maribor University Medical Centre, Slovenia

*CORRESPONDENCE
Samar J. Melhem
K1831594@kingston.ac.uk

SPECIALTY SECTION
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

RECEIVED 20 October 2022
ACCEPTED 27 February 2023
PUBLISHED 14 March 2023

CITATION
Melhem SJ, Nabhani-Gebara S and Kayyali R (2023) Latency of breast cancer stigma during survivorship and its influencing factors: A qualitative study. *Front. Oncol.* 13:1075298. doi: 10.3389/fonc.2023.1075298

Latency of breast cancer stigma during survivorship and its influencing factors: A qualitative study

Samar J. Melhem*, Shereen Nabhani-Gebara and Reem Kayyali

School of Life Sciences, Pharmacy and Chemistry, Kingston University London, Kingston upon Thames, United Kingdom

Introduction: Breast cancer diagnosis and treatment have been shown in studies to have a negative impact on patients' physical, psychological, and social well-being, as well as overall quality of life. Psychologically, it's linked to sadness, anxiety, and demoralisation. Stigma contributes to the hidden burden of breast cancer as a chronic illness. Research on the elements that breast cancer survivors encounter as influences on stigma associated to the disease is lacking. Based on the lived experiences of breast cancer survivors, this study sought to investigate the factors that lead to the manifestations of both self- and public breast cancer stigma.

Methods: Individual semi-structured interviews with 24 patients diagnosed with breast cancer were performed, followed by five focus groups with 25 patients diagnosed with breast cancer. Interviews were verbatim transcribed and analysed using thematic framework analysis.

Results: Two major themes have emerged from the data: a) Breast cancer stigma among breast cancer survivors, highlighting the various manifestations of stigma and the variables that influence them; including disease-related factors, patients' views of cancer, public perceptions of breast cancer, family and interpersonal dynamics, and b) Stigma resilience and empowerment, emphasising the necessity of sociocultural transformation and coping strategies to preserve resilience.

Conclusions: To improve the well-being of breast cancer survivors, practitioners and health policymakers should be aware of the breast cancer stigma that underpins patients' emotional and behavioural outlooks and its potential consequences on patients' quality of life. They need to develop interventions to address the different stages of cancer stigma taking into consideration sociocultural influences, norms, and beliefs.

KEYWORDS

breast cancer, stigma, survivor, manifestations, Arab, Middle East, Jordan, factors (individual factors, contextual factors)

Introduction

Breast cancer is the most common female cancer worldwide and particularly in the Middle East (1). Breast cancer is Jordanian women's most prevalent malignancy and the leading cause of cancer-related death (2). According to Jordan's Cancer Registry (JCR), breast cancer is the most common cancer among females 38.5%, accounting for around 20.8% of all cancer diagnoses (2). Breast cancer in Jordan, similar to other low- and middle-income neighbouring countries, has a variety of distinguishing characteristics. The median age at presentation is 52 years, ten years younger than the median age in developed countries. Additionally, about a third of patients present with locally advanced or metastatic disease, highlighting the critical nature of early detection programmes (3).

Breast cancer diagnosis and treatment have been proven in studies to have a significant detrimental impact on patients' physical, psychological, and social well-being, as well as and overall quality of life (4). Psychologically, it's connected to depression, anxiety, and demoralisation in cancer patients (5). Breast cancer treatment modalities typically include a combination of surgery, chemotherapy, radiotherapy, targeted or hormonal therapy (6). Nearly every breast cancer patient has a surgical resection as part of their cancer treatment, which may cause disfigurement or changes in body image. Patients are stigmatised regardless of whether they have mastectomy or breast conserving surgery (4, 7). Throughout the course of the disease, the psychosocial burden of the disease and its ramifications such as poor social support, role functioning issues, and family crisis may further contribute to stigmatisation (8). As a result of recent breakthroughs in cancer treatment modalities and quality of care, the life-changing burden of cancer has shifted from treatment and mortality rates to a spectrum of medical and non-medical issues known as survivorship (9). Cancer survivors are confronted with a plethora of cancer treatment's long-term adverse effects that may be a culprit in breast cancer stigmatisation. The most prevalent symptoms of which include fatigue, vasomotor symptoms, sexual dysfunction, musculoskeletal symptoms, neuropathy, and cognitive changes. Further, they may potentially develop cancer-induced conditions like osteoporosis, cardiac toxicity, obesity, infertility, and secondary malignancies (10). Breast cancer management as a chronic disease necessitates a focus on stigma as a contributor to the illness's hidden burden (11). Stigma is a term that refers to a sense of isolation, exclusion, devaluation, and criticism that occurs throughout a social process or personal experience and has an impact on the results of physical, psychological, and social adjustment (4). Disease-related stigma is "the stigmatisation of an illness, which may be directed towards an individual or a group of people with the illness." (5) It has detrimental consequences from diagnosis to completion (12), and to some extent, it will deter patients from seeking medical help, causing them to frequently attempt to conceal their condition from others and delay seeking medical care (13). Internalisation of cancer-related stigma results in low self-esteem and poor mental health, smaller social networks and less social potential to obtain support, and increased anticipation of social rejection, all of which negatively impact quality of life (5). Cancer is considered a socially stigmatised disease in many cultures, but some aspects of stigma are more prevalent than others (14). Previous research suggested a pervasive stigma associated with cancer. According to an early study, 52% of women with breast cancer in the United States of America reported feeling avoided or dreaded (14). Recent research has proven the high prevalence of cancer stigma in the U.S. and across many nations, including but not limited to, Japan, England, and Korea (15–18). Marlow and Wardle (19) used a web-based questionnaire to investigate public attitudes toward cancer patients in the United Kingdom. The researchers defined policy opposition (e.g., more government funding spent on cancer care and treatment) and financial discrimination as unfavourable reactions to cancer patients in addition to avoidance (e.g., It is acceptable for banks to refuse to make loans to people with cancer). Similarly, Balmer et al. (20) investigated how non-cancer adult populations in high-income nations react to cancer, concluding that individuals frequently avoid discussing it. Because cancer causes fear, healthy people rarely bring it up in ordinary conversation. Typically, cancer is rarely discussed until it becomes a personal concern (21). Existing studies mostly focus on prevention, access/barriers to medical care, and treatment experience, with few evaluating cancer stigmata during survivorship. As medical treatment has improved dramatically over the past few decades, a growing proportion of cancer patients are now survivors. More research on the long-term health effects of cancer stigma is required to fully understand what patients go through from cancer diagnosis through survivorship (14). Moreover, whereas previous literature focused on the disease itself as a stigma that affected all individuals equally, it is now more pertinent to analyse cancer-related characteristics such as cancer type, visibility, and the likelihood that the disease will impede the individual's ability to achieve personal goals or interact in social contexts. A modern concept of cancer-related stigma should focus on cancer survivors as "targets," with cognizance of how others see them, their interpretations social settings, and their motives and goals (22).

There is a dearth of research examining the most prevalent aspects and influencing factors of breast cancer-related stigma experienced by breast cancer survivors. In this study, we use qualitative methods to bridge this gap and offer new insights that can be used to empower health programme developers and policymakers by identifying crucial areas in which this population could benefit from education to change perceptions and dispel misinformation and by developing culturally relevant breast health promotion programmes for Jordan and comparable Middle Eastern Arabic cultures.

Methods and materials

Study design

The study used semi-structured interviews and focus groups to collect qualitative data as part of a larger project on breast cancer survivors' experiences and a patient-centred holistic digital platform for cancer supportive care. The qualitative methodology was chosen

to examine of cancer survivors' lived experiences, attitudes, and behaviours (23). Semi-structured interviews were used to obtain women's experiences and perspectives (24, 25). Focus groups were used to explore detailed attitudes and experiences of participants, revealing similarities and differences as perspectives and views were shared (26). The interview schedule was based on *a priori* framework and a review of studies examining experiences with breast cancer survivors. The topic guide was influenced by the Trajectory of Breast Cancer (TBC) framework developed by Simit et al. (27), who synthesised stories from a heterogeneous group of women with different types of breast cancer. They varied in age, disease stage, treatment regimen, country of origin, and other characteristics (27). This review's scope allowed for a broad analysis of international breast cancer narratives. It provided a breast cancer timeline so researchers to consider breast cancer experiences relative to TBC time-points. Specifically, the framework included the time-point 'survivorship,' which is important given the rising number of breast cancer survivors (27). This highlights the complicated illness of breast cancer and the need for support before, during, and after active treatment (5). The topic guide covered three sections: the first section explored cancer challenges and the patient journey; the second section focused on communication with healthcare professionals, treatment challenges, and follow up; and the third section explored social support sources, care pathway constraints, coping strategies, and online resources for adjusting to the illness. The interview guide includes prompts. A pilot interview with a breast cancer survivor was audiotaped and transcribed verbatim before the study began to ensure clarity, flow, format, and structure of the questions. This interview was excluded from the final analysis. See the supplementary document for the full interview guide.

Ethical considerations

This qualitative study was approved by Jordan University Hospital (JUH) approval number: (10/2019/8990) and Kingston's university ethical requirements for scientific research (Approval number/1416). Before the study, participants gave written and verbal consent. Specifically, verbal consent was obtained from members participating in online group discussions.

Data collection

Participants were recruited from oncology outpatient clinics at Jordan University Hospital, a large tertiary semi-governmental hospital in Amman. Purposeful sampling was used to reflect the whole population of breast cancer survivors by identifying those articulate, reflective, and willing to share their experience with the interviewer (28). Inclusion criteria were: 1) diagnosis of primary breast cancer with or without recurrence from all stages (I-IV), 2) adult female aged 18 and over, 3) completed curative or salvage therapy (i.e., in follow up stage or surveillance) and 4) cognitively able to comprehend the implications of consent and participate in an interview or focus group. Two breast oncologists identified 73 patients for the study. The cancer nurse specialist called them and invited them to participate. The nurse sent them a predesigned participant information sheet (PIS) on the study's aims and objectives *via* WhatsApp. Nine patients declined to participate. The lead female investigator (SJM), who has considerable experience in qualitative research, contacted the 64 patients who joined to discuss the interviews and address any queries. Participants were also assured of the anonymity and confidentiality of all information collected for the study. Participants' demographics and medical records were collected before interviews/focus groups. The investigator (SJM) assigned participants a 1:1 interview or a focus group. Thirty-three patients preferred an interview while 31 were scheduled for a focus group. Before conducting the interviews, 15 patients dropped out, leaving 24 for the semi-structured 1:1 interview, and 25 participated in five online focus groups. Except for participants 4 and 24, who participated in a focus group., all were interviewed once (29) The data coding continued until theoretical saturation was reached and no new concepts were discovered by repeated reviewing and coding. After codes were complete, themes were developed. Themes were saturated after 22 interviews and 4 focus groups. All scheduled interviews and focus groups were held as planned (30, 31). Face-to-face Interviews were conducted between (2/1/2020 and 28/2/2020) and lasted from 42-147 minutes (average time 67 minutes). During the interviews, no one else was present. Participants were informed of the research's purpose and signed consent forms were collected before the interviews. Focus groups were held online between (30/4/2020 and 18/6/2020) *via* Skype due to COVID-19 restrictions. For focus groups, verbal consent was obtained from all members before starting. Flexible question order and probing questions were used to explore some issues in depth.

Data analysis

All interviews were audiotaped in Jordanian Arabic, transcribed verbatim by the first author (SJM) and later translated to English (SJM). Two bilingual colleagues (RK and SN-G) who are fluent in Arabic and English reviewed the translated transcripts. Field notes were only taken after a 1:1 interview. No transcripts were returned to participants for feedback who were unknown to the interviewer. The study used framework methodology, a form of qualitative thematic analysis that integrates five interconnected steps to provide a systematic and rigorous audit route (32–34). The framework approach to data analysis entails five stages: familiarisation with all data, establishment of a thematic framework (based on *a priori* objectives, concerns, and topics expressed by participants), indexing (systematic coding of the data), charting (grouping the data thematically in charts and comparing within and across participants), and mapping and interpretation (exploration of the themes in relation to overarching patterns and explanation of the findings) (32). The framework methodology is compatible with deductive and inductive reasoning, and Gale et al. (32) advocate a combined approach. Deductive approaches use pre-selected themes and codes based on prior literature, pre-existing beliefs, or the

research question, while inductive approaches derive themes from data through open (unrestricted) coding and theme refinement. According to Gale et al. (32), a mixed strategy is optimal when the research attempts to discover unanticipated aspects of the participants' experience or how they assign meaning to phenomena while exploring some specific challenges. This paper uses a hybrid and will focus on two overarching themes related to breast cancer stigmatisation during survivorship, as revealed by the 1:1 interview and focus groups. The "health stigma and discrimination framework" was used as a theoretical framework to analyse emerging data on breast cancer stigma (5). To establish initial themes, one author (SJM) coded all transcripts. Two authors (RK and SN-G) contributed to the conceptualization and development of the thematic framework and consistency review of emerging themes and codes. Regular researcher meetings ensured rigour of preliminary codes. Overarching themes are organised into specific themes and subthemes based on data analysis (35, 36). To provide "referential adequacy" and "tell the story" and show data analysis conclusions, quotations were used throughout the text. This reveals the researchers' understanding, differentiates the research participants' voices from the researchers', and allows readers to build their own interpretations (35, 37). All quotes are cited as interview/FG (survivor number, age in years). To evaluate, apply, and synthesise study results, the Standards for Reporting Qualitative Research SRQR was used (38).

Results

Participants

In total, 49 women with median age at diagnosis was 46 (range, 32 to 65) participated in interviews or focus groups. Twenty-nine were married, 11 were single, 7 were divorced/separated, and 2 were widowed. Most participants were diagnosed in stages I or II, and the length of survivorship ranged from 1 to 23 years. Most of the study population was highly educated with (12/49) having a master's or doctorate degree and (24/49) holding an undergraduate degree. The characteristics of the participants are listed in Tables 1, 2.

Themes

Two overarching themes emerged from the data 1) Breast Cancer Stigma experiences during survivorship (Figure 1); 2) Stigma resilience and empowerment mechanisms (Figure 2).

Theme 1: Breast cancer stigma experiences during survivorship

The stigmatisation process during survivorship involves a complex interaction of disease-related issues, patients' views of the illness, sociocultural factors, interpersonal and family relationships, all of which contribute to the stigma of breast cancer as a "lived reality." Three subthemes were identified in relation to breast cancer-related stigma manifestations throughout

TABLE 1 Breast cancer survivors' interviews (n=24).

ID	Age at Diagnosis (Years)	Age (years)	Education‡	Marital Status	No. of children	Occupation	Family history/Gene positivity	Cancer Stage	Treatment modality◊	Trajectory of care	Years of survivorship
1	44	51	undergraduate	married	3	nurse	no	II	S^R+C+R+H	follow up	7
2	37	49	undergraduate	single	0	office administrator	yes	I	S^c+C+R+H	follow up	12
3	51	58	undergraduate	married	3	housewife	no	II	S^R+C+T	follow up	7
4**	49	55	undergraduate	married	3	self-employed	no	II	S^R+C+R+T	follow up	6
5	43	48	masters	separated	2	housewife	yes	II	S^R+C+R+T	follow up	5
6	40	45	secondary	married	1	housewife	unknown	II	S^R+C+R+T	follow up	5
7	46	52	undergraduate	married	2	teacher	yes	II	S^R+C+R+H	follow up	6
8	43	44	secondary	married	3	housewife	unknown	II	S^R+C	Follow up	1
9	39	43	undergraduate	separated	2	teacher	yes	II	S^R+C+R+H	follow up	4
10	43	45	undergraduate	single	0	government employee	yes	0	S^c+R+H	follow up	2
11	40	43	undergraduate	married	3	teacher	yes	II	S^R +C+R+H	follow up	3

(Continued)

TABLE 1 Continued

ID	Age at Diagnosis (Years)	Age (years)	Education‡	Marital Status	No. of children	Occupation	Family history/Gene positivity	Cancer Stage	Treatment modality◊	Trajectory of care	Years of survivorship
12	47	52	secondary	married	6	housewife	no	II	S^{R} +C+R+H	follow up	5
13	51	54	undergraduate	married	3	teacher	yes	II	S^{R}+C+R+H+T	follow up	3
14	55	67	secondary	married	7	retired/teacher	unknown	III	S^{c}+C^{neo}+R+T	follow up	12
15	49	55	masters	separated	1	pharmacist	no	II	S^{c}+C+R+H	follow up	6
16	49	57	doctorate	married	4	academia (linguistics)	yes	II	S^{w}+C^{neo}+R+H	follow up	8
17	36	38	undergraduate	single	0	temporary unemployed	no/genetic mutation	III Triple negative	S^{R}+C	follow up	2
18	60	73	general education	married	4	housewife	unknown	II	S^{R}+C+R+H	follow up	13
19	42	45	undergraduate	married	2	unemployed (Tourism)	yes	II	S^{c}+C+R+H	follow up	3
20	60	71	undergraduate	single	0	nutritionist	unknown	II	S^{c}+C+R+H	follow up	11
21	40	49	undergraduate	married	5	housewife	no	IV	S^{R}+C +R+H+2n line chemo	follow up	9
22	32	37	undergraduate	single	0	non-governmental organisation (NGO)	no/genetic mutation	I	Bilateral mastectomy +C+R+H	follow up	5
23	43	46	undergraduate	separated	2	civil engineer	no	I	S^{R}+C+R+H	follow up	3
24*	35	37	doctorate	divorced	0	psychologist/social worker	no	I	S^{c}+C+H	follow up	2

‡ (39) https://www.scholaro.com/pro/Countries/Jordan/Education-System.
◊ SR, Modified Radical Mastectomy.
Sw, wide local excision.
Sc, Breast conserving surgery.
R, Radiotherapy.
C, Chemotherapy.
Cneo, Neoadjuvant chemotherapy.
H, Hormonal therapy.
T, Targeted therapy.

10.3389/fonc.2023.1075298

TABLE 2 Breast cancer survivors' focus groups (n=25).

ID	Age at Diagnosis (Years)	Age (years)	Education‡	Marital Status	No. of children	Occupation	Family history/ Gene positivity	Cancer Stage	Treatment modality◊	Trajectory of care	Years of survivorship
FG1_S1	42	44	doctorate	single	0	nurse/academic	yes/genetic mutation	II	S^{c}+C+R+H	under treatment (aesthetics)	2
FG1_S2*	35	37	doctorate	divorced	0	psychologist/social worker	no	I	S^{c}+C+R+H	follow up	2
FG1_S3	52	58	secondary	married	3	housewife	no	III	C^{neo}+S+T	follow up	6
FG1_S4	41	46	masters	married	4	math teacher	yes	I	S^{c} +C+R	follow up	5
FG2_S1	44	52	masters	married	3	government employee (civil engineer)	yes	I	S^{c}+C+H	follow up	8
FG2_S2	57	68	general education	married	7	housewife	no	I	S^{w}+C	follow up	11
FG2_S3	50	54	undergraduate	married	2	retired (lab technician)	no	II	S^{R}+C+R+T	follow up	4
FG2_S4	49	55	undergraduate	married	3	self-employed	no	II	S^{R}+C+R+T	follow up	6
FG3_S1	50	56	undergraduate	married	3	housewife	no	II	S^{R}+C+H	follow up	6
FG3_S2	32	39	undergraduate	divorced	0	software engineer	yes	II	S^{R}+C+R+H	Follow up	7
FG3_S3	51	54	secondary	Married	5	housewife	No	III	S+C^{neo}+R+T	Follow up	3
FG3_S4	58	64	general education	widowed	0	cooking from home	No	II	S^{R}+C+R+H+T	Follow up	6
FG3_S5	62	67	secondary	widowed	4	housewife	No	II	S^{R}+C+R+T	Follow up	5
FG3_S6	48	57	masters	single	0	nutritionist	No	II	S^{w}+C+R+H+T	Follow up	9
FG4_S1	52	59	masters	single	0	retired nurse	Yes	II	S^{R}+C+R+H	Follow up	7
FG4_S2	60	73	secondary	married	0	housewife	No	II	S^{R}+C+H	Follow up	13
FG4_S3	38	42	undergraduate	married	1	pharmacist	No	I	S^{c}+C+R+H	Follow up	4
FG4_S4	65	69	secondary	married	2	housewife	No	III	S^{R}+C+R+H+T	Follow up	4
FG4_S5	38	41	undergraduate	married	0	human resources	Yes	II	S^{R}+C+R+H	Follow up	3
FG5_S1	35	38	secondary	single	0	administrative clerk	No	II	S^{R}+C+R+H	Follow up	3
FG5_S2	48	71	undergraduate	married	5	retired (social worker)	No	I	S^{R}+C+R+H	Follow up	23
FG5_S3	51	56	undergraduate	married	4	retired/Government (legal affairs)	No	II	S^{R}+C+R+H	Follow up	5
FG5_S4	48	58	masters	married	2	retired/banker	No	I	S^{c}+R+H	Follow up	10

(Continued)

10.3389/fonc.2023.1075298

10.3389/fonc.2023.1075298

TABLE 2 Continued

ID	Age at Diagnosis (Years)	Age (years)	Education‡	Marital Status	No. of children	Occupation	Family history/ Gene positivity	Cancer Stage	Treatment modality◊	Trajectory of care	Years of survivorship
FG5_S5	42	45	undergraduate	single	0	accounting auditor	No	II	S^{c}+C+R+H	Follow up	3
FG5_S6	41	43	masters	single	0	computer engineer	Yes	I	S^{c}+R+H	Follow up	2

‡ (39) https://www.scholaro.com/pro/Countries/Jordan/Education-System.
◊ SR, Modified Radical Mastectomy.
Sw, wide local excision.
Sc, Breast conserving surgery.
R, Radiotherapy.
C, Chemotherapy.
Cneo, Neoadjuvant chemotherapy.
H, Hormonal therapy.
T, Targeted therapy.

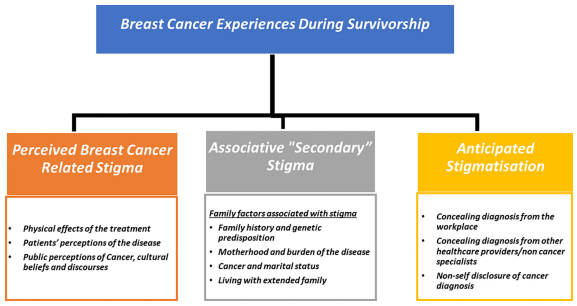


FIGURE 1
Breast cancer stigma during survivorship.

survivorship: a) perceived cancer - related stigma (PCS) b) secondary stigma or associative stigma; and c) anticipated stigmatisation. The quotations in Boxes 1-4 provide further evidence for these themes.

Perceived cancer-related stigma (PCS)

The subtheme, perceived breast cancer-related stigma during survivorship, referred to how cancer survivors' perceptions (attitudes, beliefs, and experiences) are impacted by a variety of influential factors and circumstances that contribute to a stigma latency during survivorship. These issues include the physical effects of cancer treatment (visible short-term effects of treatment-induced physical changes such as alopecia, nail changes, skin changes, and post-surgical sequelae), long term effects of treatment, and the failure or inadequacy of financial assistance for aesthetic and reconstructive surgery.

> *"I lost my hair after the second cycle. I didn't expect how swiftly it would fall out. Afterward, I cried so much, and I broke the mirror. My whole family, even my husband, came to my side to help me feel better. I remember that time; I'm glad it's over. You might think that after 13 years, all of the negative effects will have gone away, but it will still leave scars in one's heart." Interview 1 (S1, 51)*
>
> *"I took the mirror out of the bathroom because just looking at myself reminds me of my condition, it's like a disease signature.". FG1 (S5, 41)*

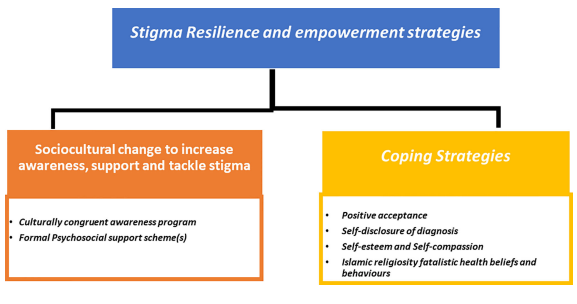


FIGURE 2
Stigma resilience and empowerment strategies.

BOX 1 Perceived cancer-related stigma (PCS).

Physical effects of the treatment

1.1. "When my doctor told me that even if he performed reconstruction surgery, my breasts would never be the same as before, I burst into tears, but I reminded myself that this was the best we could do. He told me that you are fortunate because many women are not candidates for reconstructive surgery. So, I'd never be the same as I used to be. The illness alters your personality. Not just one thing, but several." Interview 8 (S8, 44)

1.2. "I was beautiful and athletic before cancer, but I can't seem to get back into shape. As a result of Tamoxifen, I gained a lot of weight and experienced hot flashes; my doctor advised me to continue taking it for at least 7 years or the cancer would return. It's giving me a lot of side effects, but I have to take it because my fear of cancer returning is so strong that if I don't have to go through chemotherapy again, you better kill me, and I'm feeling helpless." Interview 19 (S19, 45)

Patients' perceptions of the disease

1.3. "Seeing another cancer patient having chemotherapy or looking like a cancer patient will remind you of yourself and your experience; other people will not know or comprehend how the sickness transforms us from the inside out. Interview 4 (S4, 55)

1.4. "In the beginning, everyone told me that this was a dangerous disease, and I needed to undergo regular check-ups... " I used to feel a lump in my breast when I put my hand there. Of course, I was very afraid, and my nerves were very tired. I was on edge at the time." Interview 12 (S12, 52)

1.5. "My greatest concern is that the disease would recur, not because I am terrified of death, but because I do not want to start over with treatments, particularly chemotherapy, which is excruciating and dreadful." Interview 14 (S14, 67)

1.6. "Cancer, in general, makes you fragile and emotional; it also changes your perspective on life; you begin to see things differently; we keep it to ourselves. FG4 (S4, 69)

Society's perception of the disease and cultural beliefs and discourses

1.7. " On Facebook, people only discuss cancer after someone dies from it; in our culture, they only mention two points: someone had cancer and, after a while, she lost her struggle with cancer; they don't even use the word cancer." FG1 (S2, 37)

1.8."I know they're not intentionally trying to make me feel bad, but I can no longer go to the funerals of my family members who have died. I don't know how I became this way; before my cancer diagnosis, I was a very gregarious person, but I can't stand by and see others grieve or go to funerals. People who are close to me won't understand, and social norms and duties make me feel like I'm a prisoner of this illness. I often wait 3 or 4 days before attending sorrowful events since my heart can't handle it". FG4 (S1, 59)

1.9. "I know God will reward me for all the suffering. I start to think about it and it makes me feel bad. I know that our lives are in God's hands.". Interview 16 (S16, 57)

> *"I'm sure someone will notice something wrong when they look at me. I used to be sensitive, but not any longer. This is a naive way of thinking; cancer problems extend much beyond body image. For example, my relationships with others have changed; I used to have many friends, but now I only have a few since they don't understand what it's like to live with this condition for a long period". Interview 7 (S7, 52)*

Other quotes related to the physical effects of the treatment are provided in box 1.1. Several participants reported experiencing lymphedema, osteoporosis, a lack of strength, and pain as the most common long-term physical side effects of the treatment. They believe that these side effects are inevitable and that they have little control over them, which leaves them feeling helpless.

> *"Since I've been sick for nine years, I'm feeling weak and tired. My bones hurt, my arm hurts, my sight isn't what it used to be, and every part of my body hurts. I can't tell anyone because they'll be bored, and they won't get me. The doctors won't be able to fix everything even if I come to the hospital. Some people say that this condition can be treated, but the effects can't be treated." Interview 21 (S21, 49)*

Younger survivors in reproductive age (24/49 survivors, ≤ 45 years at the time of diagnosis) indicated that the long-term effects of treatment, particularly the adverse effects of hormone medication, robbed them of their feeling of youth and femininity. They gained weight and felt and looked older than their actual age.

> *"My period stopped four years ago after I finished my therapy. The doctors informed me it could be because of the therapy, which has made me frustrated; I no longer feel like a young lady."*

BOX 2 Associative "secondary stigma".

2.1. **"**I don't know who is going to take care of my autistic child, I am the only one who can take care of him, crying." Interview 8 (S8, 44)

2.2. "When I got the disease, my little child was 2 years old and my daughter was 7,now my daughter is 17 and can take care of her brother if I'm gone, I'm grateful to God and my doctor that I was able to survive despite all the suffering, I told my doctor I didn't want to get chemo again.so he prescribed a mouth pill, we decided not to tell anyone it had returned, and my children have no idea what it means. I don't want them to hear about the condition from other people because they are still young children." Interview 21 (S21, 49)

2.3. "I don't talk much about it with my family as long as I am ok. We only share positive thoughts, no one like to talk about something related to cancer, I am concerned about my daughters, I don't want them to experience what happened to me, it's a brutal disease. Interview 13 (S13, 54)

2.4. "My ex-husband was quite supportive during therapy; now, after four years, he has married another; I simply informed him that I wish to divorce". Interview 5 (S5. 48)

2.5. "I am divorced, my family is upset because we see marriage as a long-term commitment, no one in our family is divorced, I have no choice, and I am unable to begin a new relationship." FG3 (S2, 39)

2.6. "I was diagnosed when I was 32 years old; I am still alive, but I have lost many things; my mother and brother are aware of my disease; my father has another wife, and we are a large family; they are unaware……. "I had both of my breasts removed. Because everyone in our small town knows everyone else, many of whom are family, we didn't tell anyone; I'm not sure if they did a genetic test, but my doctor advised me to get both breasts removed. I didn't have the energy to inquire why at the time." Interview 22 (S22, 37)

BOX 3 Anticipated stigmatisation.
3.1. "I took annual vacation and didn't tell anyone at work except my manager, who was quite encouraging and I told her to keep it a secret." Interview 7 (S7, 52)
3.2." When I was diagnosed I started planning my life for the next 5 years and I told my sons, I thought it's hopeless and I decided to retire early since the treatment takes a long time and I don't want to be in this working environment, and I didn't tell anybody". FG5 (S4,58)
3.3."I just didn't tell anyone except a few close friends and family members because I knew they would talk about it behind my back and hurt us. I can't take any more emotional pain because I'm already full." Interview 16 (S16, 57)

FG 5 (S6, 43)

Several participants expressed that, despite their eligibility for breast reconstruction or having a prosthesis, they could not afford it, in contrast to the costs of therapy, which are covered by the Ministry of Health. In addition, prothesis failure and its complications exacerbated the issue and hindered the patients' ability to maintain or improve their body image.

> *"I just wished the doctor would show me a picture of how my breast will look after mastectomy or a video; I don't like how it looks, but I have to accept it. My doctor has told me that reconstruction is an option, but that surgery is out of the question for me. When I asked, they told me that the Ministry of Health only covers treatment costs, and that reconstruction is not a treatment." Interview 8 (S8, 44)*
> *"Having a silicon implant to boost my self-esteem and confidence didn't work for two years, so now I'm going to the clinic to get rid of the problems that came from it." Interview 19 (S19, 45)*

Additionally, patients' perceptions of the illness, which include (cancer identity and alienation; different experiences and emotions associated with cancer; cancer as a vicious cycle and religious fatalism), contributed to perceived cancer-related stigma.

As a group, cancer survivors share a common experience because of their body changes. Some participants claimed that having cancer gives them a new identity, which has been associated with social detachment or alienation, such as not participating in sad ceremonies or funerals, avoiding people and not actively interacting with peer patients in an effort to avoid adopting this identity.

> *"I don't like going to the cancer centre because you just see cancer patients; it depresses me; that's why I chose to be treated here in the hospital, because there are other patients like diabetics and arthritis, not just cancer." FG1 (S1, 44)*

According to cancer survivors, the disease was associated with cancer-specific feelings and emotions (fear, distress, anxiety, sadness, and despair). These views originate from treatment, incapacitation, recurrence, and death, and might be reinforcing elements in the disease's perceived stigmatisation.

> *"When I hear of someone who lost their battle with cancer or who had a recurrence, it reminds me of myself and my journey, and how difficult it was, ……, I still feel sad for this sad ending." Interview 16 (S16, 57)*
> *"Yes, as you progress, you'll feel better and your morale will rise, especially when they say you're cured and everyone is happy after finishing the treatment stage, yes, the big fears and worries go away, but there is still an entrenched fear of cancer returning, which is why I come here for follow-ups every three months Interview 23 (S23, 46)*

BOX 4 Stigma resilience and empowerment.
Coping strategies
Islamic religiosity's fatalistic health views and behaviour
4.1. "I always remind myself how much God cares about me.", Interview 15 (S15, 55)
4.2 "Because our ages and our lives are all in God's hands, we should be faithful, which is very vital, I mean acceptance and faith, will be half the way to mental relief and wellbeing. Though the diagnosis itself is a major milestone, life goes on and we must cope." FG 5 (S2, 71)
4.3."I see this struggle as a blessing in disguise because it brought me closer to God. He is the only one who knows the pain, and he will pay me back in the afterlife." Interview 2 (S2, 49)
4.4. "I like to be around people, and I don't care what they think of me because I become more self-conscious. Since my diagnosis, my life has been guided by a hadith from the Prophet Mohammed. (Never a believer is stricken with a discomfort, an illness, an anxiety, a grief or mental worry or even the pricking of a thorn but Allah will expiate his sins on account of his patience)." FG5 (S2, 71)
Sociocultural change to increase awareness, support and tackle stigma
4.5. "Before I had the disease, I used to think like every one, every one fears cancer, our society think that all cancers are the same, it leads to death eventually, every year we see this ultra-positive pink campaign "promise us you will get checked", we hear lots of positivity like it will be highly curable, but when I get into the experience the society still fear the disease, they don't know what does it mean to go through all this, they don't know how our lives changed upside down, we have awareness campaigns but we need support programs and the awareness programs should focus on the rest of the journey not only screening and treatment." FG5 (S4, 58)
4.6. "It's going around like the flu! When I come to follow up, I see new women coming in to check for symptoms; nearly every one of us knows someone who has the disease; we have a screening programme, but I believe it is insufficient; I believe we need more effective awareness programmes to educate the public, family, and caregivers; and these initiatives should be tailored to our cultural and societal norms." FG1 (S2, 37)
4.7."We can't make national support programmes or support groups until we fix cultural problems. If we don't, many women won't join…"FG1 (S1, 44)

> *"I don't feel down all the time, but I become really worried between follow-ups, especially if there are new results, or if I have certain symptoms, such as adverse effects, or if I am waiting for an imaging report, waiting for test results is stressful and overwhelming" Interview 4 (S4, 55)*

Other informants viewed cancer as an empty circle that will come back.

> *"The doctor told me it was triple negative, but I didn't understand what that meant at the time, they don't tell you everything, just drop by drop, I came here to the clinic because I finished chemotherapy 3 months ago and they ran a PET scan, the doctors discovered a likely spread in the pelvis, I don't know what to say, I don't want to stay at home because my family is even more upset than me, especially my sister who knew everything from the beginning, it's an empty circle." Interview 17 (S17, 38)*

Additional quotes related to patients' perceptions of the disease are provided in Box 1.

According to the participants, societal perceptions of cancer and cultural ideas and discourses such as the ingrained nature of cancer fear, exhibiting pity as an inferiority signal instead of compassion, and society's prejudice and judgmental unfavourable stance on cancer all play a role in internalising cancer stigma.

> *"I didn't tell anyone about my condition because I didn't want to have that look of pity in their eyes that truly hurts, that they feel sorry for me." FG 5 (S4, 58)*

Further, some cancer survivors' outlook on the disease was influenced by their religious beliefs. Fatalism is the belief that one's health is beyond one's ability to control. For instance, cancer fatalism is the belief that death is inevitable when cancer is present. In a religious setting, fatalism also refers to the belief that health outcomes are predetermined or under the control of a higher power, primarily God.

> *" I don't know why I got the disease, and I believe in the prophet Mohammad hadith." What is meant for us will find its way to us. What is not meant for us will never come to us. It is Allah's will, and this is my fate, I think that it's my destiny to get this disease and I can't do anything about it". Interview 21 (S21, 49)*
> *"My sisters don't care and don't want to listen to me when I told them about screening, they said that no one knows the future except God, they said they might have other kinds of cancer, there are many causes and one death." Interview 13 (S13, 54)*

Additional quotes on community's perceptions of the disease and cultural and religious beliefs influencing perceptions are provided in Box 1.

Secondary or associative stigma

The second subtheme, "associative stigma" as a manifestation of breast cancer stigmatisation, relates to the experience of stigma by cancer survivors' family members. According to the responses, associative stigma may be influenced by family history and genetic predisposition, motherhood and disease burden, cancer, and marital status, and living with extended family.

> *"When I received my diagnosis, it was the worst shock of my life; I was just 42 years old and a university doctor, and I love my job. My siblings were really supportive, and they went out of their way to help me. But there is a hidden side of the story; as I already said, I am BRACA2 positive, and my aunt died from the same condition. I'm still not sure how it will affect us socially, but as for me, I'm keeping it to myself, only few people know, and as a family, we're keeping it to ourselves." FG1 (S1, 44)*
> *"In general, people are more aware that breast cancer can spread in families. This isn't true for everyone, but only those who have been properly educated on the subject. In the end, this could affect someone's chances of getting married or her social status, because society isn't willing to change, and the collective mind can't be changed. It's because if they know that you have a gene, they might think that you're a "flawed person." FG1 (S2, 37)*
> *"I was divorced after 11 years of marriage and without children; someone wants to propose to me, and I want to be a mother; I underwent oocytes cryopreservation since the doctor told me it was the only way to get pregnant and have children in the future. However, there is a slim probability that it will be successful. I'm not sure if he'd still come back and propose. You are also aware of the changes that occur following surgery. It's difficult to put into words." Interview 24 (S24, 37)*

Further quotes on associative stigma can be found in Box 2.

Anticipated stigmatisation

The third subtheme explores the elements that contribute to the expression of anticipated stigmatisation among cancer survivors. These elements contribute to cancer diagnosis concealment in a variety of contexts as an adaptive response to identity threat or as a strategy for stigma resilience. Several survivors stated that they concealed their cancer diagnosis at work or from non-cancer healthcare providers. Additionally, several women acknowledged concealing their diagnosis from extended family members and disclosing it selectively to others.

> *"I had annual mammograms for seven years because I am educated, and I used to go with my friends. After noticing a discharge from my nipple, I rushed to the hospital, where I was referred to an oncologist. He told me to contact him if the discharge changed colour, such as if it turned bloody. When the discharge turned brown, I was terrified. Following a biopsy and an MRI, my doctor discovered ductal carcinoma in situ and performed ductal excision. Seven years were squandered performing mammograms. And they could only identify it with an MRI; I told my doctor I would have paid a thousand JD (1400*

$) to perform the test myself, he said it is not routinely required. I cried a lot after the doctor removed all of my breast tissue and removed my nipple. I asked my doctor to never use the term (cancer) in the medical report, simply the phrase "mammary ducts excision," as I do not want anyone in the workplace to know I had cancer, I want to get promoted and pursue my career." Interview 10 (S10, 45)

"During chemotherapy, I used to cover my face when I visit the cancer centre since my students are training there and I don't want them to notice me, now I only come to see the surgeon here in the clinic" FG1 (S1, 44)

"I work in the private sector; when I informed them, I wanted to start my treatment procedure, they refused to give me an unpaid vacation; they stated I could come back and apply after I recovered." Unfortunately, they hired someone else because, as you know, women in our community aren't supposed to support their families financially, but I don't want to lose my job. Then I applied for another Job, not telling them I had cancer because it was none of their business." FG5 (S6, 43)

"I can't work as long as I used to, I don't have the stamina, I can't tell my supervisor what's going on with me, like hot flashes, how my hormones and period changes are impacting me, and all my co-workers are guys." Interview 23 (S23, 46)

"I didn't feel this way because I never told anyone about my disease. I also didn't quit my job, so I'm always working." Interview 10 (S10, 45)

"I never told a dentist or other doctor that I had cancer. I didn't even tell the pharmacist when I bought a drug." Interview 10 (S10, 45)

"My husband told me not to tell anyone from his family because they might start interfering and putting pressures, my parents are old, so I only told them that it's a benign lump because my sister had breast cancer and died 15 years ago." Interview 19 (S19, 45)

Further quotes to support the sub-theme anticipated stigmatisation are provided in Box 3.

Theme 2: Stigma resilience and empowerment

Despite the wide stigma experienced and perceived, the second theme describes the strategies and adaptations used by some cancer survivors to overcome stigmatisation adversities (Figure 2). This theme includes a sub-theme on coping strategies, which covers the following: a) positive acceptance; "*Cancer has changed me profoundly, and I became a better person as a result. Because of what I've been through, I'll be more attentive to the needs of others, and I'll always do my best to get others close to me in order for them to remember how beautiful I was, because life is short and there should be something influential, we can do for others, because we're only here for a little time." Interview 9 (S9,43) b*) self-disclosure of diagnosis; "*I was diagnosed with cancer and treated at the hospital where I work. Everyone knows and has been very supportive, especially my doctor. This has given me the chance to switch to a more positive role. My doctor always calls me to support women who come to the clinic. When they see me, they cheer up and say, "Impossible! You never look as you had it before!" Interview 2 (S 2,49)* c) self-esteem and self-compassion; " *You hear a lot of stories, some of which make you sad. I tell myself how lucky I am compared to those who have advanced cases or diseases that can't be cured…… even though I'm suffering, where I've been and how strong I was when I was smashed to pieces."* Another informant said, "*I tell myself, life is beautiful," all the time. Sometimes I can't feel it, but I keep reminding myself of my blessings. If nothing is good, there is bad and there is worse. This keeps me going." Interview 15 (S15,55)* and d) Islamic religiosity's fatalistic health views and behaviour- According to some participants, this is a response to cancer as a chronic illness that brings them calm and tranquilly and aids in coping with disease adversities and social challenges.

"As I already said, the tumour was discovered in a miraculous manner. (She said, "I went to the hospital for an abdominal CT scan because I was experiencing kidney colic pain. The hospital called me two days later to tell me that they had found a lump in the lower part of my breast. That's how my story began.) When I think of how early the disease was discovered, I am reminded of God's mercy and care, there are some things in life that we can't control or predict, and we will be tested in different ways in this life and rewarded in the afterlife." Interview 24 (S24, 37)

More quotes on this sub-theme are provided in Box 4.

The other subtheme is related to sociocultural change to increase awareness, support and tackle stigma. Cancer survivors indicated that empowerment and increased engagement in support programmes necessitate a sociocultural transformation at the national level, as well as the development of formal psychosocial support services.

"No one cares about those who survive. They only get treatment…. which is good compared to other countries, but we don't have formal psychosocial programmes to help empower women and meet their needs." FG 3(S2,39)

"We need new ways to speak about cancer in the media that reflect the reality of the disease and the challenges in our society and to motivate people to react positively and become more engaged; also, many women in our country are disempowered and require support, as well as novel ways to reach out to the public." FG5 (S2, 71)

Other quotes to support this sub-theme are provided in Box 4.

Discussion

This study addressed the social construction of breast cancer-related stigma as a "lived reality" through Jordanian breast cancer survivors' narratives and experiences. We sought to comprehend self and public breast cancer stigma, as well as the elements that contribute to its manifestations, which stem from cancer survivors' conceptions, beliefs, and lived experiences with the disease as a chronic condition. Other factors include social interactions, cultural and religious beliefs, societal norms, and discourse. Two overarching themes emerged from the data. The first theme- breast cancer stigma during survivorship -

portrayed the various manifestations of cancer-related stigma during survivorship as a complex interaction of disease-related factors, family dynamics and interpersonal relationships, patient perceptions of the disease, and public perceptions and sociocultural beliefs and attitudes. Our findings revealed that PCS (5) was influenced by short- and long-term cancer-related physical consequences (for example, alopecia, mastectomy, and weight gain). Previous research indicated that regaining physical and psychological health following a breast cancer diagnosis may be a long and challenging process for survivors (especially in terms of body image issues) (4). Financial constraints to restoring a healthy body image or the failure of cosmetic procedures, according to our results, are among the factors that contribute to disease stigmatisation. A substantial body of evidence suggests that PCS adversely impacts breast cancer patients' attitudes, behaviours, psychological, and quality of life (4, 40, 41). Almost all the breast cancer patients who underwent treatment suffered from significant side effects. The majority of survivors experienced significant and long-lasting physical alterations, clinical symptoms, sleep disturbances, and lifestyle changes that reduced their quality of life (8). Therefore, survivors' inability to adjust and manage long-term effects of treatment such as weight gain, fatigue, and osteoporosis also contribute to the inability to restore physical well-being. PCS is prevalent among women with breast cancer and stigmatisation includes negative feelings and attitudes (such as frustration, despair and depressive emotions, negative attitude and decreased healthcare seeking behaviour) as well as social avoidance of survivors (42). As the disease causes physical, psychological, and social morbidity, cancer stigma has been identified as an impediment to health promotion (43). The entrenched fear of the disease at the both the individual and public level, as well as the threat of death as it is perceived as a vicious cycle due to fear of recurrence or progression, may contribute to the stigma associated with cancer. This study corroborates with previous studies indicating that breast cancer and its treatment bestow a long-term identity change and confer a "cancer identity, "which is the basis for this judgement (11). Manifestations included social isolation, avoiding other cancer patients, and fatalistic beliefs and appearance-based judgments. The stigma associated with breast cancer is a concern. PCS has negative impacts on patients and their families at home, in the community, and at work. Anticipated stigmatisation refers to the anticipation of prejudice from others if a health problem becomes known (5). This is manifested by concealing diagnosis in different situations. This can be explained by the identity threat model proposed by Kapp et al. (44) and its components "situational threat" and "personal attributions" that enable cancer survivors to evaluate potentially harmful cues in a variety of situations (e.g., the workplace, social interactions/contexts, and the healthcare system) in different ways. Disease concealment may promote resilience and adaptation by allowing cancer survivors to selectively process disease origins, features, and effects. Although stigma sensitivity due to cancer-induced physical alterations is unique from other social stigmas, its psychosocial manifestations may be conducive to social stigma. Patients who are more sensitive or conscious of being stigmatised because of their cancer may feel more threatened by their identity. The desire to protect themselves from any threats that they perceive as endangering their professional or personal development contributes to the stigma experienced by cancer survivors, which may sustain their self-esteem in the short term but perpetuate negative stereotypes in the long run (22, 44). Cancer has a unique stigma, yet there is no universal agreement that having cancer places a person in a single stigmatised group or category. Cancer survivors vary in how much they internalise their cancer identity as part of their self-identification and this may be influenced by cultural and religious beliefs about cancer's lethality, poor reputation, and taking control of one's life. Those who internalise and identify with stigmatised beliefs, whether actual or perceived, may identify more as a cancer "sufferer" than as a cancer "survivor," which conveys a positive connotation (44). The latency and dynamic nature of breast cancer-related stigma may be explained using Jones et al. (45) theory of social stigma that identified six distinguishing characteristics: concealability, course, disruptiveness, aesthetic quality, origin, and risk. "Concealability" relates to how easy patients may conceal their cancer. For example, breast cancer mastectomy is less concealable than gastrointestinal cancer. "Course" focuses on people's perceptions about cancer prognosis and death. Cancer is commonly linked with death, even though advances in cancer therapy have enabled most cancer patients (approximately 60%) to live for a considerable period following diagnosis (46). Therefore, stigmatisation varies by stage and cancer continuum (treatment vs remission). "Disruptiveness" refers to how much cancer interferes with relationships and communication. Impaired psychosocial functioning or a poor prognosis among cancer survivors may impede contact with others who are hesitant or unsure how to approach them (15, 19). "Aesthetic" refers to the degree to which cancer makes people aesthetically unpleasant. Bilateral mastectomy or unsymmetrical breasts can cause significant disfigurement. Female chemotherapy-induced alopecia may be more aesthetically unpleasant than in male patients. Aesthetic concerns were mostly expressed by younger participants in this study. "Origin" refers to whether patients are thought to be responsible for their cancer (e.g., smoking, obesity, poor diet, or lifestyle). Because breast cancer is sometimes thought to be hereditary (due to genetic susceptibility (e.g., BRACA1/2 germline and family history)) (3), having a family member with cancer makes people feel as if their feeling of security is jeopardised or that they are not immune to cancer.

Associative or secondary stigma refers to the experience of stigma by relatives or friends of stigmatised group members or among healthcare practitioners who offer treatment to stigmatised group members (5). This was also identified among breast cancer survivors and is consistent with Arab culture. Arab women feared their husbands would divorce them or take a second wife if they were no longer fertile or sexually attractive, compromising family integrity (46). Protecting and promoting the well-being of their families was a top priority for many Jordanian and Arab women. Some mothers hide their diagnoses from their children to spare them sadness. Our findings suggest internalised stigma may undermine mothers' protective sensations, especially in extended families (47, 48). In their sociocultural context, it is not uncommon for Arab women who are aware of a family history or genetic predisposition to assume that their daughters or sisters have a lower likelihood of marrying or having children and to blame themselves. As a result, people prefer not to share anything that could jeopardise or stigmatise their family's integrity or reputation (47–49), and families frequently respond to social stigma and its attendant harm to family well-being by maintaining strict concealment regarding

breast cancer (47–49). This can lead to delays in seeking medical evaluation or early detection (50). This is of concern considering that the median age of the study population is relatively young.

The second theme describes stigma resilience and empowerment based on sociocultural change and coping strategies used by survivors to counter the stigmatisation process. Our findings show that empowered breast cancer survivors may play a key role in promoting early detection by sharing positive experiences of successful breast cancer survivorship. Cultural awareness programmes should go beyond early diagnosis to shed light on the survivorship phase, improve public understanding of "survivorship" identity, debunk misconceptions, and provide specific psychological support initiatives to boost survivors' health wellbeing. Awareness campaigns must be tailored to the different sociocultural norms and religious standards of the community or subcommunities, as well as the specific needs of survivors. It should reduce fear and stigma and encourage routine screenings. The goal of cancer education is to increase people's knowledge of the causes and prognosis of breast cancer, addressing its progression, aetiology, disruptiveness, and risk (14). Further, the media is a key cultural venue that promotes illness-related structural stigma, such as in the workplace, so transformative reforms are needed in how cancer topics are communicated to the public. Self-compassion, positive acceptance of diagnosis, and self-disclosure of illness are partially aligned with earlier diagnosis and improve resilience (51). Islamic religion and religious fatalism were also good influences on resilience; this is congruent with research on Islamic philosophy of disease. In this respect, breast cancer was seen to be a test of faith delivered by God (47, 52). To pass this exam, survivors interpreted it as a call for tolerance, patience and acceptance. Such behaviour would be forever rewarded through the atonement of sins and reward in the hereafter ("*Al-Akhira*").

Study strengths and limitations

The study was conducted in one hospital. Despite this, Jordanian cancer survivors are typically treated in multiple treatment facilities due to resource constraints. As a result, their interactions with the entire healthcare system may be reflected in their experiences. limitations of the study were that it only included Jordanian cancer survivors who were eligible for government treatment funding schemes. Non-Jordanians with cancer were not included, even though some are refugees with greater unmet needs due to existing socioeconomic inequities. Moreover, the study included a homogeneous group of cancer survivors, most of whom were in stages I and II. Only two survivors with incurable metastatic disease underwent salvage chemotherapy for stages III and IV. For advanced incurable cancers, further research is warranted as the stigmatisation process varies with the course of disease progression (i.e. early vs. relapse or metastatic) cancer.

Conclusions

Breast cancer-related stigma is a latent and hidden burden of the disease during survivorship, according to this study's findings. Health policy makers and oncology practitioners need to allocate more attention and resources to reducing cancer-related stigma among the public and creating psychosocial supportive programmes for cancer survivors who are at risk of stigmatisation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

This qualitative study was approved by Jordan University Hospital (JUH) approval number: (10/2019/8990) and Kingston's university ethical requirements for scientific research (Approval number/1416). Before the study, participants gave written and verbal consent. Specifically, verbal consent was obtained from members participating in online group discussions. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: SJM, RK, and SN-G; Methodology: SJM, RK, and SN-G; Data collection and transcriptions: SJM; Formal analysis: SJM, RK, and SN-G; Writing and Editing: SJM and RK; Supervision and Revision: RK and SN-G. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1075298/full#supplementary-material

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2021) 71(3):209–49. doi: 10.3322/caac.21660

2. World Health Organisation, International Agency for Research on Cancer and Jordan Globocan. (2020). Available at: https://gco.iarc.fr/today/data/factsheets/populations/400-jordan-fact-sheets.pdf (Accessed on 28 January 2022).

3. Abdel-Razeq H, Abujamous L, Abunasser M, Edaily S, Bater R. Prevalence and predictors of germline BRCA1 and BRCA2 mutations among young patients with breast cancer in Jordan. *Sci Rep* (2021) 11(1):14906. doi: 10.1038/s41598-021-94403-1

4. Wang QX, Bai Y, Lu GF, Zhang CY. Perceived health-related stigma among patients with breast cancer. *Chin Nurs Res* (2017) 4(4):158–61. doi: 10.1016/j.cnre.2017.10.002

5. Stangl AL, Earnshaw VA, Logie CH, van Brakel W, C Simbayi L, Barré I, et al. The health stigma and discrimination framework: a global, crosscutting framework to inform research, intervention development, and policy on health-related stigmas. *BMC Med* (2019) 17(1):31. doi: 10.1186/s12916-019-1271-3

6. Swainston K, Campbell C, van Wersch A, Durning P. Treatment decision making in breast cancer: a longitudinal exploration of women's experiences. *Br J Health Psychol* (2012) 17(1):155–70. doi: 10.1111/j.2044-8287.2011.02028.x

7. Waljee JF, Ubel PA, Atisha DM, Hu ES, Alderman AK. The choice for breast cancer surgery: can women accurately predict postoperative quality of life and disease-related stigma? *Ann Surg Oncol* (2011) 18(9):2477–82. doi: 10.1245/s10434-011-1582-x

8. Suwankhong D, Liamputtong P. Breast cancer treatment: Experiences of changes and social stigma among Thai women in southern Thailand. *Cancer Nurs* (2016) 39 (3):213–20. doi: 10.1097/NCC.0000000000000255

9. Moore HCF. Breast cancer survivorship. *Semin Oncol* (2020) 47(4):222–8. doi: 10.1053/j.seminoncol.2020.05.004

10. Stan D, Loprinzi CL, Ruddy KJ. Breast cancer survivorship issues. *Hematol Oncol Clin North Am* (2013) 27(4):805–27. doi: 10.1016/j.hoc.2013.05.005

11. Weiss MG, Ramakrishna J, Somma D. Health-related stigma: rethinking concepts and interventions. *Psychol Health Med* (2006) 11(3):277–87. doi: 10.1080/13548500600595053

12. Liang D, Jia R, Yu J, Wu Z, Chen C, Lu G. The effect of remote peer support on stigma in patients after breast cancer surgery during the COVID-19 pandemic: A protocol for systematic review and meta-analysis. *Med (Baltimore)* (2021) 100(24): e26332. doi: 10.1097/MD.0000000000026332

13. Ndukwe EG, Williams KP, Sheppard V. Knowledge and perspectives of breast and cervical cancer screening among female African immigrants in the Washington D.C. metropolitan area. *J Cancer Educ* (2013) 28(4):748–54. doi: 10.1007/s13187-013-0521-x

14. Fujisawa D, Hagiwara N. Cancer stigma and its health consequences. *Curr Breast Cancer Rep* (2015) 7(3):143–50. doi: 10.1007/s12609-015-0185-0

15. Phelan SM, Griffin JM, Jackson GL, Zafar SY, Hellerstedt W, Stahre M, et al. Stigma, perceived blame, self-blame, and depressive symptoms in men with colorectal cancer. *Psychooncology* (2013) 22(1):65–73. doi: 10.1002/pon.2048

16. Lebel S, Devins GM. Stigma in cancer patients whose behaviour may have contributed to their disease. *Future Oncol* (2008) 4(5):717–33. doi: 10.2217/14796694.4.5.717

17. Cho J, Choi EK, Kim SY, Shin DW, Cho BL, Kim CH, et al. Association between cancer stigma and depression among cancersurvivors: A nationwide survey in Korea. *Psychooncology* (2013) 22(10):2372-8. doi: 10.1002/pon.3302

18. Fujisawa D, Fujimori M, Basaki-Tange A, Umezawa S, Ueda- Nouno J, Adachi A, et al. (2014). Perceived stigma and social discrimination among cancer survivors in Japan–a web-based survey, in: *American Psycho-social Oncology Annual Meeting*, Tampa, FL, USA, 2, 15–7.

19. Marlow LA, Wardle J. Development of a scale to assess cancer stigma in the non-patient population. *BMC Cancer* (2014) 14:285. doi: 10.1186/1471-2407-14-285

20. Balmer C, Griffiths F, Dunn J. A qualitative systematic review exploring lay understanding of cancer by adults without a cancer diagnosis. *J Adv Nurs* (2014) 70 (8):1688–701. A review on understanding of cancer in non-cancer population. doi: 10.1111/jan.12342

21. Peters-Golden H. Breast cancer: varied perceptions of social support in the illness experience. *Soc Sci Med* (1982) 16(4):483–91. doi: 10.1016/0277-9536(82)90057-0

22. Major B, O'Brien LT. The social psychology of stigma. *Annu Rev Psychol* (2005) 56:393–421. doi: 10.1146/annurev.psych.56.091103.070137

23. Bryman A. *Social research methods. 5th ed*. Oxford, United Kingdom: Oxford University Press (2016).

24. Geertz C. *The interpretation of cultures*. New York, NY: Basic Books (1973).

25. Carspecken FP. *Critical ethnography in educational research: A theoretical and practical guide*. Routledge. (1996).

26. Freeman T. 'Best practice' in focus group research: Making sense of different views. *J Adv Nurs* (2006) 56(5):491–7. doi: 10.1111/j.1365-2648.2006.04043.x

27. Smit A, Coetzee BJ, Roomaney R, Bradshaw M, Swartz L. Women's stories of living with breast cancer: A systematic review and meta-synthesis of qualitative evidence. *Soc Sci Med* (2019) 222:231–45. doi: 10.1016/j.socscimed.2019.01.020

28. Tinati R, Halford S, Carr L, Pope C. Big data: Methodological challenges and approaches for sociological analysis. *Sociology* (2014) 48(4):663–81. doi: 10.1177/0038038513511561

29. Vasileiou K, Barnett J, Thorpe S, Young T. Characterising and justifying sample size sufficiency in interview-based studies: systematic analysis of qualitative health research over a 15-year period. *BMC Med Res Methodol* (2018) 18(1):148. doi: 10.1186/s12874-018-0594-7

30. Hennink M, Kaiser BN. Sample sizes for saturation in qualitative research: A systematic review of empirical tests. *Soc Sci Med* (2021) 292:114523. doi: 10.1016/j.socscimed.2021.114523

31. Guest G, Namey E, McKenna K. How many focus groups are enough? building an evidence base for nonprobability sample sizes. *Field Methods* (2017) 29(1):3–22. doi: 10.1177/1525822X16639015

32. Gale N, Heath G, Cameron E, Rashid S, Redwood S. Using the framework method for the analysis of qualitative data in multi-disciplinary health research. *BMC Med Res Methodol* (2013) 13(1):117. doi: 10.1186/1471-2288-13-117

33. Ritchie J, Lewis J. *Qualitative research practice: A guide for social science students and researchers*. London: Sage (2003).

34. Parkinson S, Eatough V, Holmes J, Stapley E, Midgley N. Framework analysis: a worked example of a study exploring young people's experiences of depression. *Qual Res Psychol* (2016) 13(2):109–29. doi: 10.1080/14780887.2015.1119228

35. Koch LC, Niesz T, McCarthy H. Understanding and reporting qualitative research: An analytical review and recommendations for submitting authors. *Rehabil Couns Bull* (2014) 57(3):131–43. doi: 10.1177/0034355213502549

36. Kline WB. Developing and submitting credible qualitative manuscripts. *Counselor Educ Supervision* (2008) 47(4):210–7. doi: 10.1002/j.1556-6978.2008.tb00052.x

37. Elliott R, Fischer CT, Rennie DL. Evolving guidelines for publication of qualitative research studies in psychology and related fields. *Br J Clin Psychol* (1999) 38(3):215–29. doi: 10.1348/014466599162782

38. O'Brien BC, Harris IB, Beckman TJ, Reed DA, Cook DA. Standards for reporting qualitative research: a synthesis of recommendations. *Acad Med* (2014) 89 (9):1245–51. doi: 10.1097/ACM.0000000000000388

39. *Education system in Jordan*. Available at: https://www.scholaro.com/pro/Countries/Jordan/Education-System (Accessed 29/11/2022).

40. Ernst J, Mehnert A, Dietz A, Hornemann B, Esser P. Perceived stigmatization and its impact on quality of life-results from a large register-based study including breast, colon, prostate and lung cancer patients. *BMC cancer* (2017) 17(1):1–8. doi: 10.1186/s12885-017-3742-2

41. Amini-Tehrani M, Zamanian H, Daryaafzoon M, Andikolaei S, Mohebbi M, Imani A, et al. Body image, internalized stigma and enacted stigma predict psychological distress in women with breast cancer: A serial mediation model. *J Advanced Nursing* (2021) 77(8):3412–23. doi: 10.1111/jan.14881

42. Mosher CE, Danoff-Berg S. Death anxiety and cancer-related stigma: a terror management analysis. *Death Stud* (2007) 31(10):885–907. doi: 10.1080/07481180701603360

43. Corrigan P. How stigma interferes with mental health care. *Am Psychol* (2004) 59(7):614–25. doi: 10.1037/0003-066X.59.7.614

44. Knapp S, Marziliano A, Moyer A. Identity threat and stigma in cancer patients. *Health Psychol Open* (2014) 1(1):2055102914552281. doi: 10.1177/2055102914552281

45. Jones EE. *Social stigma: The psychology of marked relationships*. WH Freeman (1984).

46. Allemani C, Weir HK, Carreira H, Harewood R, Spika D, Wang XS, et al. Global surveillance of cancer survival 1995–2009: analysis of individual data for 25,676,887 patients from 279 populationbased registries in 67 countries (CONCORD-2). *Lancet* (2015) 385(9972):977–1010. doi: 10.1016/S0140-6736(14)62038-9

47. Fearon D, Hughes S, Brearley SG. Experiences of breast cancer in Arab countries. a thematic synthesis. *Qual Life Res* (2020) 29(2):313–24. doi: 10.1007/s11136-019-02328-0

48. Dewan MF, Hassouneh D, Song M, Lyons KS. Development of the breast cancer stigma scale for Arab patients. *Asia Pac J Oncol Nurs* (2020) 7(3):295–300. doi: 10.4103/apjon.apjon_14_20

49. Jassim GA, Whitford DL. Understanding the experiences and quality of life issues of bahraini women with breast cancer. *Soc Sci Med* (2014) 107:189–95. doi: 10.1016/j.socscimed.2014.01.031

50. Solikhah S, Matahari R, Utami FP, Handayani L, Marwati TA. Breast cancer stigma among Indonesian women: a case study of breast cancer patients. *BMC Womens Health* (2020) 20(1):116. doi: 10.1186/s12905-020-00983-x

51. Aizpurua-Perez I, Perez-Tejada J. Resilience in women with breast cancer: A systematic review. *Eur J Oncol Nurs* (2020) 49:101854. doi: 10.1016/j.ejon.2020.101854

52. Sadati AK, Lankarani KB, Gharibi V, Fard ME, Ebrahimzadeh N, Tahmasebi S. Religion as an empowerment context in the narrative of women with breast cancer. *J Relig Health* (2015) 54(3):1068–79. doi: 10.1007/s10943-014-9907-2

53. Education system in Jordan. Available at: https://www.scholaro.com/pro/Countries/Jordan/Education-System (Accessed 14/5/2022).

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 25 May 2023
DOI 10.3389/fonc.2023.927748

OPEN ACCESS

EDITED BY
Ariella Hanker,
University of Texas Southwestern Medical Center, United States

REVIEWED BY
Caroline Aquino Moreira-Nunes,
Federal University of Ceara, Brazil
Regis Resende Paulinelli,
Universidade Federal de Goiás, Brazil

*CORRESPONDENCE
Jane Rocha Duarte Cintra
janerdc@terra.com.br

RECEIVED 24 April 2022
ACCEPTED 01 May 2023
PUBLISHED 25 May 2023

CITATION
Ferreira AdSS, Cintra JRD, Fayer VA, Nogueira MC, Júnior CB, Bustamante-Teixeira MT, Chaoubah A, Cintra AD, Simão CM and Guerra MR (2023) Breast cancer survival and the health system in Brazil: an analysis of public and private healthcare.
Front. Oncol. 13:927748.
doi: 10.3389/fonc.2023.927748

Breast cancer survival and the health system in Brazil: an analysis of public and private healthcare

Adriana de Souza Sérgio Ferreira[1,2], Jane Rocha Duarte Cintra[1]*, Vívian Assis Fayer[3], Mário Círio Nogueira[3], Cassimiro Baesso Júnior[4], Maria Teresa Bustamante-Teixeira[2,3], Alfredo Chaoubah[2,3], Arthur Duarte Cintra[1,5], Caroline Montes Simão[1,5] and Maximiliano Ribeiro Guerra[3]

[1]Instituto Oncológico de Juiz de Fora/Hospital 9 de Julho, Departamento de Oncologia Clínica, Juiz de Fora, Brazil, [2]Programa de Pós-Graduação em Saúde da Universidade Federal de Juiz de Fora (UFJF), Faculdade de Medicina, Juiz de Fora, Brazil, [3]Programa de Pós-Graduação em Saúde Coletiva da Universidade Federal de Juiz de Fora (UFJF), Departamento de Saúde Coletiva, Juiz de Fora, Brazil, [4]Hospital Universitário da Universidade Federal de Juiz de Fora (UFJF), Juiz de Fora, Brazil, [5]Faculdade de Ciências Médicas e da Saúde de Juiz de Fora (SUPREMA), Juiz de Fora, Brazil

Background: The incidence of breast cancer is increasing globally; however, survival outcomes vary and are lower in developing countries.

Methods: We analyzed the 5- and 10-year survival rates for breast cancer according to the type of healthcare insurance (public *vs*. private) in a referral center for cancer care in the Brazilian southeast region. This hospital-based cohort study included 517 women diagnosed with invasive breast cancer between 2003 and 2005. The Kaplan–Meier method was used to estimate the probability of survival, and the Cox proportional hazards regression model was used to assess prognostic factors.

Results: The 5- and 10-year breast cancer survival rates were as follows: private healthcare service survival rate of 80.6% (95% CI 75.0–85.0) and 71.5% (95% CI 65.4–77.1), respectively, and public healthcare service survival rate of 68.5% (95% CI 62.5–73.8) and 58.5% (95% CI 52.1–64.4), respectively. The main factors associated with the worst prognosis were lymph node involvement in both healthcare services and tumor size >2 cm only in public health services. The use of hormone therapy (private) and radiotherapy (public) was associated with the best survival rates.

Conclusions: The survival discrepancies found between health services can be explained mainly by the difference in the stage of the disease at the time of diagnosis, indicating inequalities in access to the early detection of breast cancer.

KEYWORDS

survival analysis, cohort study, breast neoplasm, prognosis, neoplasm staging

Background

Among the malignant neoplasms that affect women, breast cancer (BC) is associated with high morbidity and mortality worldwide (1), including in Brazil (2). Although BC incidence remains high in high-income countries, these countries have already experienced a tendency to reduce mortality, while middle- and low-income countries show an increased incidence with still high BC mortality (1). Combining population screening with advances in cancer treatment has been identified as an important factor in reducing mortality and the consequent expansion of the number of survivors in high-income countries (3), which reinforces the understanding that the existing differences in availability and access to early cancer diagnosis and treatment contribute to justifying the disparity observed between regions (3, 4).

The relative 5-year BC survival in Brazil increased from 68.7% between 2000 and 2004 to 75.2% between 2010 and 2014, according to surveillance data produced by the CONCORD-3 study. These percentages are lower than those found in North America and Oceania, which have values close to 90% (5). Meanwhile, the 5-year BC survival in Brazil, estimated through hospital-based studies in recent decades, has ranged from 75% to 87% (6–12), while the 10-year BC survival has ranged from 41% to 78.7% (7, 10, 12–14).

Survival analysis is widely used in oncology, especially in BC assessment, because it provides information on the effectiveness of diagnosis and treatment. Furthermore, when performed using population data, it can contribute to identifying specific characteristics of disease behavior and its prognostic factors (14, 15).

Prognostic factors are fundamental in supporting the adoption of adequate criteria for therapeutic approaches. Staging (6, 16, 17), tumor size, lymph node status (6, 9, 11, 13), and hormone receptor (HR) status (8, 11) are classic BC prognostic factors, for which there is sufficient scientific evidence to support their strong association with survival. Individual characteristics, such as age at diagnosis (18), race (13, 17, 19), and socioeconomic profile (20, 21), and those related to health services, such as therapeutic approaches (11), access, and type of health services (public or private) (22–25), have also been identified as prognostic factors that can influence BC survival.

Since 1988, Brazil has had a public health system, the Sistema Único de Saúde (SUS), which has recognized health as a right and works through a universal system delineated by territories and hierarchical networks at integrated care levels (26). In addition, private health services (individual or corporate plans) serve 24.5% of the Brazilian population, composed mainly of formal workers who are part of corporate health insurance plans (26, 27). In the context of BC, some evaluations indicate that there are differences between public and private health services regarding diagnosis and treatment in Brazil, suggesting that public health service users present with a more advanced stage of BC at diagnosis and, consequently, have a worse prognosis (12, 25, 28).

Considering the relevant role of health services in cancer care, this study aimed to evaluate the 5- and 10-year BC survival rates according to the type of healthcare service (public *vs.* private) in a reference center in the Zona da Mata Mineira, Minas Gerais State, Brazil.

Method

This hospital-based cohort study included women diagnosed with BC between January 2003 and December 2005 who underwent surgical and/or complementary treatment (chemotherapy, radiotherapy, or hormone therapy).

All women were assisted at a regional oncology referral center located in the city of Juiz de Fora, Minas Gerais, Brazil. In 2019, the municipality presented a population estimate of 568,873 inhabitants (29), which is the hub of healthcare in the southeast macro-region of Minas Gerais State, comprising 94 municipalities, and considered as a reference in the diagnosis and treatment of several medical specialties (30). The oncology reference center where the study was conducted provides care for the public health system (SUS) and the private healthcare system, which is accredited by the High Complexity Assistance Unit in Oncology (UNACON) with radiotherapy, chemotherapy, and cancer surgery services (31).

Data from the institution's Hospital Cancer Registry were used to recruit information from patients. Data collection was carried out in a standardized form through a review of medical records by a team previously trained and advised by specialists in pathology and oncology. The retrieval of information on the follow-up of women to access vital status (defined as a determination of date of death or date last known alive) was obtained from the consultation of hospital records, the National Mortality Information System, National Registries of the Deceased (32), and the Individual Taxpayer's Registry (33), in addition to a telephone call made by the institution's Hospital Cancer Registry and contact with the patients' mastologist. Among the 563 women identified, 45 patients with carcinoma *in situ* and one who died less than 30 days after diagnosis were excluded. We analyzed 517 women with invasive cancer, which corresponded to the study population.

The evaluated variables included three dimensions: 1) sociodemographic variables, such as age at diagnosis (<50, 50–60, ≥60 years), skin color (white and non-white), and education level (high/medium, low); 2) tumor aspects: tumor size (≤2 cm, >2 cm), lymph node involvement (present or absent), stage (initial—I, intermediate—II, advanced—III and IV), hormone receptor (positive, negative, not evaluated), and expression of biomarkers such as hormone receptors (HRs) (estrogen and/or progesterone) and human epidermal growth factor receptor-type 2 (HER2) [yes = $HR^{\pm}$ and $Her2^{+}$ or HR^{+} and $Her2^{-}$; no = HR^{-} and $Her2^{-}$ (triple-negative tumor subtype)]; and 3) characteristics related to health services, such as performing tumor immunohistochemical expression according to the St. Gallen surrogate classification for breast cancer subtypes (34) (done, not done), the average time between diagnosis and first treatment (in days), type of surgery (conservative or radical), and chemotherapy/radiotherapy/hormone therapy use (no, yes). The 5- and 10-year overall BC survival rates were calculated using the time interval between the date of the histopathological report and the date of death or the end of follow-up. Women who remained alive at the end of the follow-up and follow-up losses on the date of the last contact were censored at 60 months (for a 5-year analysis) or 120 months (for a 10-year analysis). All deaths were treated as failures. To assess differences in the distribution of variables, the χ^2 test was used; when necessary,

Fisher's exact test was used. For the survival estimates and their comparison in relation to the studied variables, the Kaplan–Meier method and the log-rank test were used. The Cox proportional hazards regression model was used to assess prognostic factors by computing the hazard ratio (HR) and the corresponding 95% confidence interval (95% CI). The variable selection for the modeling process was based on clinical relevance and its statistical significance in the univariate analysis, considering the same variables for adjustment at 5 and 10 years in both services (public and private). The variables included in the multiple analyses were removed using the backward elimination process.

All analyses were performed using the Stata software package (version 16.0, StataCorp, TX, USA), and the research was approved by the Research Ethics Committee of the Federal University of Juiz de Fora (reference no. 2.038.397). The level of statistical significance was set at 5%. The quality of adjustment was assessed based on the likelihood ratio and overall measure of adjustment quality.

Results

Of the 517 women evaluated, 248 (48.1%) were assisted in private healthcare and 269 (51.9%) in public healthcare. Table 1 shows the distribution of women according to the study variables stratified by the type of health service assistance (private or public).

The most frequent characteristics found in both services were as follows: age group over 60 years, white skin color, residence in the municipality of the regional oncology referral center (Juiz de Fora), underwent tumor immunohistochemical expression, first treatment within 15 days of diagnosis, intermediate stage (II), tumor size >2 cm, positive hormone receptors, identified biomarker expression, and had undergone chemotherapy, radiotherapy, and hormone therapy (Table 1).

Among the women assisted in public healthcare, in the 10-year follow-up, higher percentages of death (39%, $p < 0.001$), low schooling level (63%, $p < 0.001$), non-white skin color (31.5%, $p < 0.001$), tumor size >2 cm (69.1%, $p < 0.001$), lymph node involvement (49.2%, $p < 0.05$), and advanced stage (III and IV) (37.3%, $p < 0.001$) were observed, compared with those assisted by private healthcare.

Considering the characteristics related to healthcare services, having not performed immunohistochemical tumor expression (23.1%, $p < 0.001$) and the use of hormone therapy (40.1%, $p = 0.04$) were more frequent in the public healthcare service than in the private healthcare service. No significant differences were identified in relation to the type of surgery and chemotherapy or radiation therapy according to the type of health service. The average time between diagnosis and first treatment was 11.78 days (95% CI 1.76–21.80) in the private healthcare service and 18.6 days (95% CI 10.34–26.87) in the public healthcare service.

Regarding the biological aspects of the tumor, significant differences were observed between public and private health services concerning positive hormone receptors (73.1% *vs.* 65.8%, $p = 0.005$), whereas no significant difference was observed in the absence of any qualified tumor biomarkers (triple-negative tumor subtype) (21.5% *vs.* 16.3%, $p = 0.16$).

The overall 5-year BC survival rates were 80.6% (95% CI 75.0–85.0) in the private health service and 68.5% in the public health service (95% CI 62.5–73.8), respectively, while the 10-year BC survival rates were 71.5% (95% CI 65.4–77.1) and 58.5% (95% CI 52.1–64.4), respectively.

Table 2 shows the 5- and 10-year BC survival rates according to the type of healthcare for the study variables. Unadjusted survival function estimates that indicated better survival ($p < 0.05$), at 5 and 10 years, were observed among white women with tumors <2 cm, initial (I) and intermediate (II) stages, positive hormone receptors, and who underwent conservative surgical treatments and hormone therapy. The survival percentages of these characteristics in private health services were greater than those in public health services.

In the first 5 years of follow-up, the absence of an immunohistochemical profile was associated with lower survival among women from both health service types (Table 2). In this condition, the overall 5-year BC survival was 65.9% (95% CI 45.9–80.0, $p < 0.05$) in private health services and 56.6% (95% CI 42.8–68.3, $p < 0.05$) in public health services, in which 23.1% had not performed the immunohistochemical profile, a percentage significantly higher than that found in the private health service (12.5%, Table 1).

In the public health service, better 5- and 10-year survival rates were found among women who received radiotherapy (76% and 64%, respectively). Such a difference was not observed in women who were assisted in private health services.

In private health services, the prognostic factors independently associated with the risk of death, at 5 and 10 years, were the stage at diagnosis and the use of hormone therapy. While advanced staging (III and IV) was associated with an increased risk of death (5 years: HR = 11.4; 95% CI 3.75–34.9; 10 years: HR = 7.87; 95% CI 3.40–18.2), the use of hormone therapy had a protective effect (5 years: HR = 0.34; 95% CI 0.17–0.67; 10 years: HR = 0.38; 95% CI 0.21–0.66). In addition, non-white skin color was associated with a higher 10-year risk of death (HR = 1.14; 95% CI 1.01–4.96), with a trend toward a higher 5-year risk (HR = 2.23; 95% CI 0.88–5.67).

Having undergone radical surgery and the absence of hormone receptors and HER2 were associated with a higher risk of death only in the univariate analysis.

Regarding the public health service, an almost five-fold increased risk of death was observed at 5 and 10 years among women with advanced staging (HR = 4.66) compared with the initial staging. A significantly higher risk of death was also found among non-white women, when compared with white women, in 5 years (HR = 1.98; 95% CI 1.19–3.28) and 10 years (HR = 1.91; 95% CI 1.23–2.97). Hormone therapy and radiotherapy maintained the protective effect throughout the entire evaluation period, indicating that women treated with these treatments survived longer than those who were not treated with these therapeutic modalities (hormone therapy—5 years: HR = 0.28; 10 years: HR = 0.44; radiotherapy—5 years: HR = 0.47; 10 years: HR = 0.52). In addition, the use of chemotherapy reduced the risk of death by 50% at 10 years (HR = 0.51; 95% CI 0.29–0.89).

10.3389/fonc.2023.927748

TABLE 1 Distribution of study variables according to the type of healthcare services for the hospital-based cohort.

	Private		Public		
Variables	n^a	%	n^a	%	p^b
	248	48.0	269	52.0	
Status in 10 years					
Alive	182	73.4	164	61.0	<0.001
Dead	66	26.6	105	39.0	
Age					
<50	68	27.4	100	37.2	0.07
50–60	61	24.6	57	21.2	
>60	119	48.0	112	41.6	
Location					
Juiz de Fora	135	54.4	144	53.5	0.88
Other cities	113	45.6	125	46.5	
Skin color					
White	220	88.7	183	68.0	<0.001
Non-white	19	7.7	84	31.2	
Education					
High/medium	164	66.1	92	34.2	<0.001
Low	48	19.4	140	52.0	
Immunohistochemical tumor pattern[c]					
Done	218	87.5	207	76.9	<0.001
Not done	31	12.5	62	23.1	
Tumor size					
≤2 cm	113	45.6	80	29.7	<0.001
>2 cm	126	50.8	179	66.5	
Lymph node involvement					
Negative	145	58.5	132	49.1	0.04
Positive	96	38.7	128	47.6	
Staging					
Initial (I)	83	33.5	46	17.1	<0.001
Intermediate (II)	97	39.1	105	39.0	
Advanced (III and IV)	66	26.6	118	43.9	
Hormone receptor (HR)[d]					
Positive	181	73.0	177	65.8	<0.001
Negative	62	25.0	70	26.0	
Not evaluated	5	2.0	22	8.2	
Expression of biomarkers[e]					
Yes	200	80.7	186	69.1	0.16
No	39	15.7	51	19.0	

(Continued)

TABLE 1 Continued

	Private		Public		
Variables	n^a	%	n^a	%	p^b
	248	48.0	269	52.0	
Type of surgery					
Conservative	128	51.6	123	45.7	0.19
Radical	112	45.2	136	50.6	
Chemotherapy					
No	96	38.7	91	33.8	0.23
Yes	152	61.3	178	66.2	
Radiotherapy					
No	51	20.6	44	16.4	0.30
Yes	184	74.2	198	73.6	
Hormone therapy					
No	76	30.7	108	40.1	0.03
Yes	172	69.3	161	59.9	

[a]The total (n; %) of the variables may differ depending on the presence of missing data.
[b]Chi-square test (or Fisher's exact test, when indicated); significant if $p < 0.05$.
[c]According to St. Gallen surrogate classification for breast cancer subtypes.
[d]HR: estrogen and/or progesterone hormone receptor.
[e]Biomarkers: yes = $HR^{\pm}$ and $Her2^{+}$ or HR^{+} and $Her2^{-}$ (non-triple-negative); no = HR^{-} and $Her2^{-}$ (triple-negative).

Table 3 shows the adjusted association measures of the Cox models for the 5- and 10-year BC survival rates according to the type of health services used. The 5- and 10-year overall BC survival curves for significant variables in the univariate analysis (log-rank test) according to the type of health service are illustrated in Figures 1, 2, respectively.

Discussion

The overall 5- and 10-year BC survival rates were higher in the private healthcare service than in the public healthcare service. The advanced stage at diagnosis was the main factor independently associated with the worst prognosis in both health services. Therapeutic approaches to hormone therapy (in both health services), radiotherapy, and chemotherapy (in the public health service) were associated with better prognosis, whereas non-white race/skin color was associated with worse prognosis in both health services.

According to the international literature, these findings reinforce the differences in access to diagnosis and treatment in more vulnerable populations, such as those found in the Concord study, which was conducted on five continents and showed marked differences in 5-year BC survival between high-income countries (USA and Australia: ~90%) and low-income countries (South Africa: ~40%) (5). Other studies carried out in North American populations also reinforce that advanced stage at diagnosis, low socioeconomic status, and non-white race are associated with lower BC survival and are important determinants for identifying health disparities in this population (35–37).

Brazilian hospital-based studies, which mostly only evaluated women who were assisted in public health services, showed a 5-year BC survival equal to or greater than 75% (6–12), while the 10-year BC survival assessments pointed out a greater range of values, ranging between 41% and 78.7% (7, 10, 12–14). The 5-year BC survival rate found in these studies was higher than that obtained in the present study for women assisted in public health services (68.5%), which was not observed in private health services. To interpret these differences in survival, the higher percentage of characteristics suggestive of better BC prognosis in women who participated in some of these studies, such as earlier stages and positive estrogen and progesterone receptors, must be taken into account (7, 11). In line with the findings of the present study, a study that evaluated health inequities in BC survival in Brazil also observed a worse survival rate in women treated at the public health service compared with those treated at private health services, which was related to advanced staging at BC diagnosis in the public health service (25).

The 10-year overall survival of BC found in the public health service (58.5%) was higher than that observed in a study carried out at the SUS reference center for BC treatment in Joinville, State of Santa Catarina, in the southern region of Brazil (41%) (14). However, it was lower than the 10-year survival found in a university teaching hospital in Belo Horizonte, Minas Gerais (64.5%) (12). Again, these findings may be due to the difference in the distribution of BC stages between regions, since advanced stages were more frequent in Santa Catarina than in Minas Gerais, which concentrated higher percentages of early stages and well-

TABLE 2 Distribution of the 5- and 10-year breast cancer survival rates, according to the type of healthcare services and study variables, for the hospital-based cohort.

Variables	%	95% CI	p^a	%	95% CI	p^a	%	95% CI	p^a	%	95% CI	p^a
	Private						Public					
	5 years			10 years			5 years			10 years		
	80.6	75.0–85.0		71.7	65.4–77.1		68.5	62.5–73.8		58.5	52.1–64.4	
Age												
<50	82.3	70.9–89.5	0.44	77.6	65.5–85.8	0.14	62.9	52.6–71.5	0.09	52.9	42.5–62.3	0.22
50-60	84.9	73.1–91.9		76.8	63.3–85.9		80.3	67.2–88.6		66.2	51.7–77.2	
≥60	77.3	68.5–83.9		65.5	55.7–73.6		67.7	57.7–75.7		59.8	49.5–68.7	
Skin color												
White	82.3	76.6–86.8	0.02	73.4	66.8–78.9	0.02	74.4	67.3–80.2	<0.001	65.2	57.4–71.9	<0.001
Non-white	61.1	35.3–79.2		50	25.9–70.0		55.2	43.8–65.2		43.5	32.5–54.0	
Education												
High/medium	83.3	76.5–88.2	0.66	73.7	66.0–79.9	0.87	72.1	61.5–80.2	0.64	61	49.9–70.4	0.61
Low	80.4	65.8–89.3		72.6	56.6–83.5		68.8	60.1–75.9		57.8	48.8–65.8	
Tumor immunohistochemical expression[b]												
Done	82.6	76.8–87.1	0.02	73	66.3–78.6	0.12	71.9	65.2–77.6	0.01	59.8	52.5–66.3	0.10
Not done	65.9	45.9–80.0		62.5	42.5–77.2		56.6	42.8–68.3		54.4	40.4–66.4	
Tumor size												
≤2 cm	91.0	83.9–95.1	<0.001	83.1	74.5–89.0	<0.001	88.3	78.6–93.7	<0.001	81.1	70.2–88.4	<0.001
>2 cm	73.7	64.9–80.6		63.4	53.9–71.5		61.9	54.3–68.8		50.7	42.7–58.0	
Lymph node involvement												
Negative	87.3	80.7–91.8	<0.001	79.2	71.2–85.2	<0.001	75.2	66.6–81.8	0.10	68.9	59.7–76.3	0.01
Positive	70.9	60.5–79.1		61.4	50.5–70.6		65.5	56.5–73.0		51.3	42.0–59.8	
Staging												
Initial	95.1	87.4–98.1	<0.001	89.5	80.0–94.6	<0.001	90.6	76.9–96.4	<0.001	83.2	67.9–91.6	<0.001
Intermediate	90.6	82.7–94.9		81.1	71.4–87.8		81.2	72.1–87.6		72.9	62.7–80.8	
Advanced	47.7	34.9–59.3		35.8	24.1–47.7		49.2	39.8–57.9		36.7	27.8–45.6	
Hormone receptor (HR)[c]												
Positive	86.5	80.6–90.8	<0.001	78.9	72.0–84.4	<0.001	77.9	71.0–83.5	<0.001	64.1	56.2–70.9	0.03
Negative	65.7	52.3–76.5		53.8	39.7–65.9		57.8	45.3–68.5		56.1	43.5–66.7	
Expression of biomarkers[d]												
Yes	84.7	78.9–89.1	0.02	76.1	69.3–81.6	0.02	75.7	68.8–81.4	0.01	62.5	54.8–69.2	0.13
No	69.9	52.3–82.1		59.9	41.5–74.2		59.9	44.9–71.9		57.6	42.6–69.9	
Type of surgery												
Conservative	88.7	81.7–93.2	0.01	80.7	72.3–86.7	0.01	77.4	68.8–83.9	0.02	66	56.5–73.9	0.05
Radical	77.1	68.1–83.9		66.7	56.7–74.9		64.3	55.5–71.8		55.7	46.6–63.9	
Chemotherapy												
No	81.7	72.2–88.2	0.80	75.4	65.0–83.1	0.42	63.4	52.1–72.7	0.13	53.5	41.9–63.8	0.13

(Continued)

TABLE 2 Continued

Variables	%	95% CI	p^a	%	95% CI	p^a	%	95% CI	p^a	%	95% CI	p^a
	Private						Public					
	5 years			10 years			5 years			10 years		
	80.6	75.0–85.0		71.7	65.4–77.1		68.5	62.5–73.8		58.5	52.1–64.4	
Yes	79.9	72.5–85.5		69.5	61.2–76.4		71.1	63.8–77.2		61	53.2–67.9	
Radiotherapy												
No	86.1	73.0–93.1	0.53	77.2	62.5–86.7	0.59	53.0	36.6–67.1	<0.001	47.3	31.1–61.9	0.01
Yes	82.1	75.7–87.0		73.4	66.0–79.4		76.0	69.4–81.4		64.1	56.8–70.5	
Hormone therapy												
No	59.8	47.5–70.1	<0.001	48.3	36.0–59.6	<0.001	45.7	35.8–55.0	<0.001	40.9	31.1–50.4	<0.001
Yes	89.4	83.7–93.2		81.5	74.5–86.7		83.3	76.5–88.3		70.1	62.1–76.8	

[a]Log-rank test for each variable.
[b]According to St. Gallen surrogate classification for breast cancer subtypes.
[c]HR: estrogen and/or progesterone hormone receptor.
[d]Biomarkers: yes = $HR^{\pm}$ and $Her2^{+}$ or HR^{+} and $Her2^{-}$ (non-triple-negative); no = HR^{-} and $Her2^{-}$ (triple-negative).

differentiated tumors, characteristics associated with the best survival (12, 14, 23).

Most of the differences found in BC survival according to the type of health service are explained mainly by the difference in the disease stage when women arrived at the health service, indicating inequalities in access to the early detection of BC (12, 23). When the National Cancer Control Policy in Brazil was instituted (38) in 2005, and with its subsequent insertion in the strategic action plan

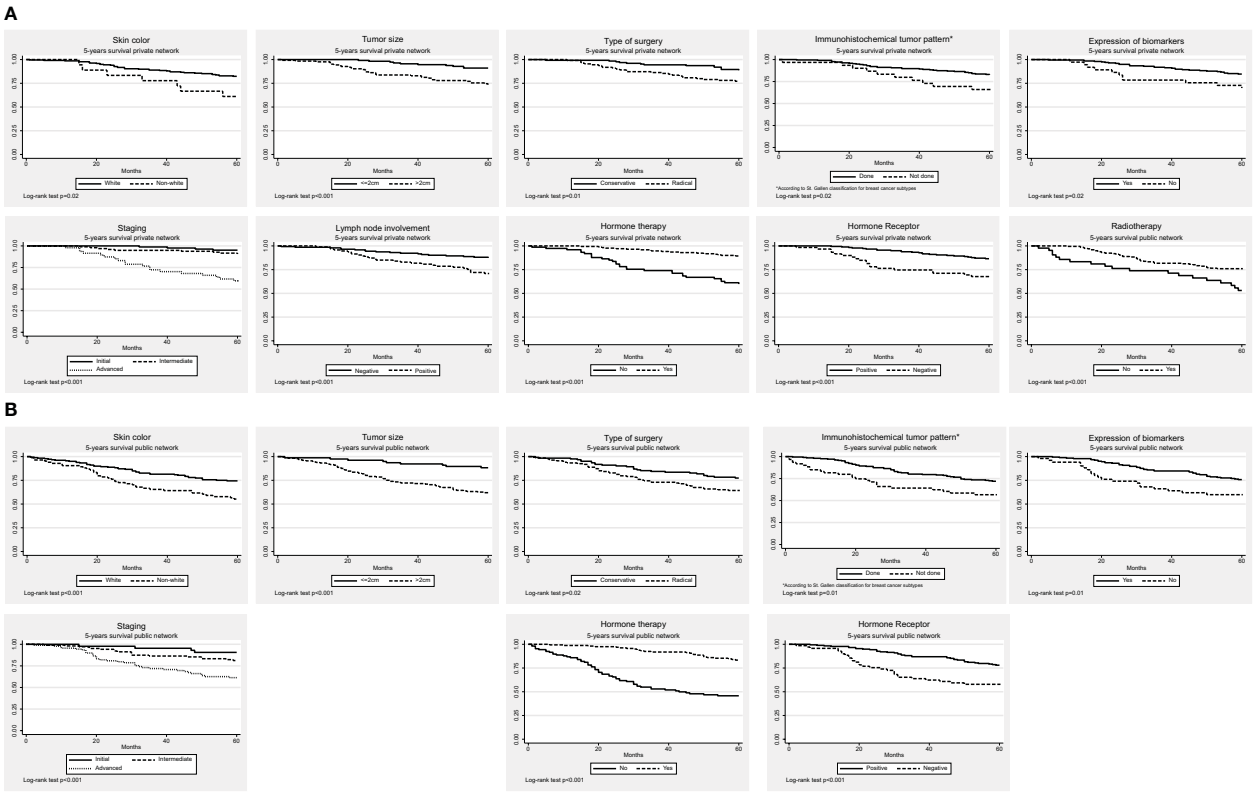


FIGURE 1
The 5-year breast cancer survival curves, according to the type of healthcare services. **(A)** Private **(B)** Public.

10.3389/fonc.2023.927748

TABLE 3 Adjusted measures of association of the Cox models for 5 and 10-year breast cancer survival rates, according to type of health care service.

	Private					
	5 years			10 years		
Variables	HR*	IC 95%	*p*	HR*	IC 95%	*p*
Staging						
Initial (I)	1			1		
Intermediate (II)	1.40	0.39–5.04	0.6	1.31	0.52–3.30	0.6
Advanced (III e IV)	11.4	3.75–34.9	<0.001	7.87	3.40–18.2	<0.001
Skin color						
White	1			1		
Non-white	2.23	0.88–5.67	0.09	2.24	1.01–4.96	0.05
Chemotherapy						
No	1			1		
Yes	1.18	0.46–3.02	0.7	1.52	0.70–3.29	0.3
Radiotherapy						
No	1			1		
Yes	0.65	0.24–1.74	0.4	0.75	0.35–1.61	0.5
Hormone therapy						
No	1			1		
Yes	0.34	0.17–0.67	0.002	0.38	0.21–0.66	0.001
	Public					
	5 years			10 years		
Variables	HR*	IC 95%	*p*	HR*	IC 95%	*p*
Staging						
Initial (I)	1			1		
Intermediate (II)	1.98	0.65–5.97	0.2	1.79	0.75–4.25	0.2
Advanced (III e IV)	4.66	1.63–13.3	0.004	4.66	2.05–10.6	<0.001
Skin color						
White	1			1		
Non-white	1.98	1.19–3.28	0.008	1.91	1.23–2.97	0.004
Chemotherapy						
No	1			1		
Yes	0.62	0.33–1.18	0.2	0.51	0.29–0.89	0.02
Radiotherapy						
No	1			1		
Yes	0.47	0.27–0.82	0.008	0.52	0.31–0.88	0.01
Hormone therapy						
No	1			1		
Yes	0.28	0.16–0.49	<0.001	0.44	0.28–0.71	0.001

HR, hazard ratio.
CI, confidence interval.
* Also adjusted for age at diagnosis (continous).

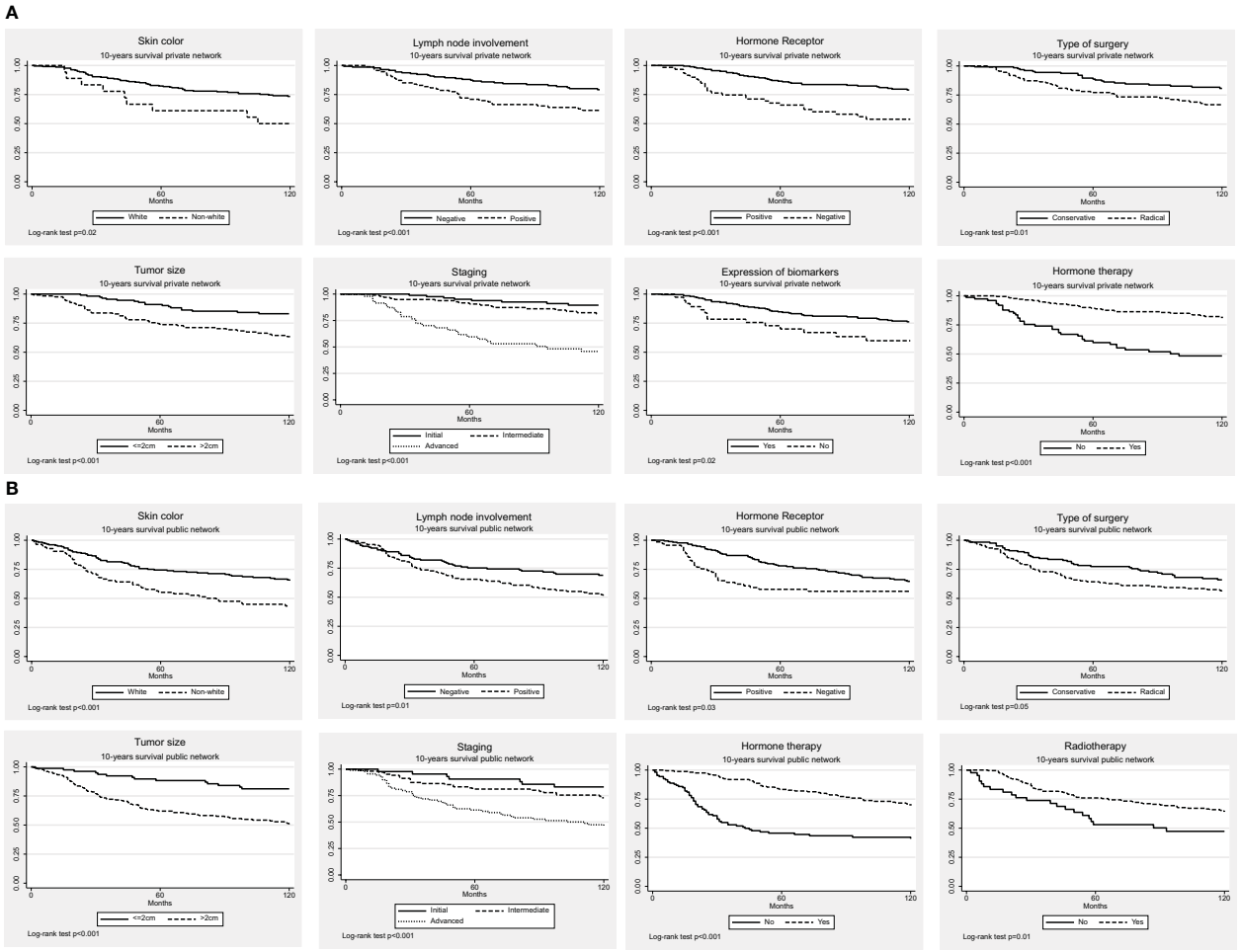


FIGURE 2
The 10-year breast cancer survival curves, according to the type of healthcare services. **(A)** Private **(B)** Public.

for coping with chronic non-communicable diseases (39), an expansion of access to mammography was observed for the age group of 50 to 69. Another important breakthrough observed was the approval of legal regulations in Brazil, which established a 30-day deadline for diagnostic confirmation and a 60-day deadline to begin treatment (40). In the present study, we observed a high percentage of women who started treatment within 30 days (over 80%) in both types of health services, indicating that access to treatment after BC diagnostic confirmation occurs in a timely manner in both services. However, obstacles in the structure and limited investments in the public cancer care network continue to harm access to early BC diagnosis, recommendations, and timely treatment. These are probably the greatest challenges to enabling a cancer control policy in Brazil that guarantees equity in access to information, tracking, diagnosis, and therapeutic approaches.

The presence of lymph node involvement and tumor size >2 cm are classic prognostic factors associated with a worse prognosis and, consequently, lower survival (6, 7, 9, 13, 14, 18). Lymph node involvement was associated with a higher risk of death in both types of healthcare services, while larger tumor size was associated with a higher risk of death only in the public health service. Women who received treatment in private health service exhibited BC at earlier stages when compared with those treated at the public health service, a finding that corroborates the results of other national studies (12, 23). Such findings suggest greater difficulty in accessing diagnostic confirmation methods and mammographic screening within the Brazilian public health service (41–43), as well as lower percentages of adherence to mammographic screening (43, 44).

The risk of death among non-white women who used the public health service was significantly higher in 5 and 10 years, which can be explained by the higher percentage of advanced stages among women being treated in the public health service. Interestingly, non-white skin color was also associated with the highest risk of death in 10 years among women treated in private health services, showing racial inequalities related to BC control, even in the private network. National and international studies that have investigated BC survival have used skin color as an indirect way of measuring 'women's socioeconomic conditions (13, 19, 25, 45–47). Such differences should consider the difficulty in accurately defining skin color due to the intense miscegenation of the Brazilian

population and the fact that, in this study, we obtained this information from the individual perceptions of health service professionals. In Brazil, Cabral et al., in their evaluation of Brazilian women with more vulnerable social profiles, such as black skin color and low education, showed long intervals between diagnosis and treatment, regardless of the stage of the disease (48). Our results corroborate those obtained in a study carried out in the southern region of the Mississippi Delta in the United States, which showed high rates of advanced stages in black women in the region, regardless of tumor subtype (20). Racial disparities were also found among African-American women when compared with European-American women, indicating that race/skin color is an important prognostic factor for BC survival. Even when tumor factors are controlled, women of African descent have a higher risk of death from BC, which suggests some secondary effects related to ethnic factors. At diagnosis, these women also have more advanced and aggressive tumors, with a disproportionate chance of survival, most likely due to inadequate access to healthcare as well as socioeconomic disadvantages (35). It is important to highlight that although tumor staging is one of the mediating factors of racial disparities in BC survival identified in several studies, it does not explain all inequalities in prognosis. Other important factors already identified are differences in treatment, the prevalence of comorbidities, and in more recent studies, the interactions between genetic and environmental factors that are mediated by epigenetic modifications (35–37). Another important aspect to emphasize is that racial disparities in BC survival are detected even in models that are also adjusted for socioeconomic variables, indicating that the race/skin color variable is not only a proxy for socioeconomic status, although the latter also plays a role in racial disparities in healthcare (19, 35–37).

The recommendation for hormone therapy was relatively high in both groups (positive hormone receptor status >65% for both health services), which may explain the better survival for women who received this therapeutic modality in both types of health services. However, a higher percentage of unevaluated hormone receptors was found in the public health service than in the private health service (8.2% public *vs.* 2.0% private; $p = 0.005$), which points to disparities in access to diagnostic and therapeutic methods between health services. Hormone therapy was also associated with a better prognosis in a study carried out on Brazilian women by Mendonça et al. (6), De Moraes et al. (7), and Guerra et al. (25).

We verified worse survival for tumors with no expression of any biomarker (triple-negative tumor subtype), corroborating the worst prognosis of this specific tumor subtype and reinforcing the need for a deeper understanding of molecular characteristics to provide more effective treatments. Similar results have been reported by Gonçalves et al. (49) comparing triple-negative and non-triple-negative tumors. In the present study, the distribution of this tumor subtype was similar between both healthcare services, which strengthens the impression that from a biological point of view, the populations under comparison were similar. In the multivariate analysis, we observed that other tumor factors independently influenced survival, such as tumor size in the public health service and lymph node involvement in both health services—factors that are related to the more advanced stage of the disease. These findings were similar to those observed by Fayaz et al. (50) in a 10-year survival study of patients with triple-negative tumors, where staging and lymphovascular invasion were the most relevant prognostic factors for the lowest survival.

Regarding the local therapeutic approach, radiotherapy was associated with better BC survival in 5 and 10 years only at public health services, which is in line with the findings of a study carried out in the western Brazilian Amazon region (11). The distribution of radiotherapy offered in the oncology care network and the displacements needed to arrive at the treatment site can partially explain the difficulties related to accessing this treatment in Brazil (51). Radiotherapy requires complex equipment infrastructure, physical facilities, and highly trained human resources so that it is offered in an appropriate way to the population, conditions that, together, limit its distribution and offer in the SUS network (51). In a study conducted in New Mexico, USA, a two-fold risk of death was identified in women who did not receive radiotherapy compared with those who used the therapies indicated by the National Comprehensive Cancer Network (NCCN) guidelines (52). The use of radiotherapy after conservative surgery reduces the rate of locoregional recurrence and the risk of death from BC, according to a meta-analysis conducted in 2011 by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), which included more than 10,000 women with pathologically negative or positive lymph nodes (53). Other studies reinforce the beneficial effect of radiotherapy after mastectomy and adjuvant chemotherapy, even among high-risk cases with lymph node involvement, large tumors, compromised surgical margins, or even in the presence of a combination of risk factors, such as age ≤50 years, triple-negative tumor, high tumor grade, and lymphovascular invasion (54, 55).

For women treated in the public health service, the protective effect of chemotherapy on long-term BC survival (10 years) was also identified. It should be noted that current chemotherapy is more effective, with a reduction in BC mortality with the use of more active regimens, especially in patients with more advanced stages of the disease, when compared with the absence (non-recommendation) of chemotherapy (56).

Although the study has limitations inherent to the use of secondary data, hospital-based cancer registries (HCRs) are recognized as important centers for collecting information on the quality of cancer care. In Brazil, accredited oncology services are required to keep HCRs active and updated, transferring information regarding the care and treatment of cancer patients to the National Cancer Institute (INCA), which uses these data to compare the quality of care provided by oncology services and promote public health policies (57). In the healthcare services where our study was conducted, the HCR has been consolidated and has been in operation since 2000. In addition, a hospital cohort allows greater access to patient follow-up, which contributes to minimizing losses of follow-up that usually occur in cohort studies; a hospital-based cohort allows the adoption of different strategies to retrieve follow-up information, contributing to minimizing the impact of these losses as well as making it possible to recover some selected socioeconomic information through telephone contact, as was carried out for all cases in this study. Although the mean follow-up time was longer in the private network than in the public network, both for the 5-year survival analysis (private: 53.6

months; 95% CI = 51.8–55.3; public: 47.8 months; 95% CI = 45.5–50.0) and 10-year survival analysis (private: 92.4 months; 95% CI = 87.7–97.1; public: 80.5 months; 95% CI = 75.2–85.8), more deaths were identified in the public network than in the private network, and loss to follow-up was not significantly different between health services. As a result of the strategies adopted to retrieve the information, we identified very few losses over 60 months (private: 4.0%; public: 7.4%) and 120 months (private: 18.3%; public: 15.2%).

Furthermore, even in the face of difficulties in evaluating therapeutic recommendations due to the scarcity of available information, the main predictive factors that could influence the use of these therapies were considered during the analysis. On the other hand, the results emphasized the importance of the information produced by health services, which makes it possible to identify the challenges faced, particularly in the public health service responsible for the cancer care of the majority of the Brazilian population (42), as well as makes it possible to produce relevant content to support BC control practices and improve service quality.

Conclusions

There was a greater BC survival rate in the private healthcare service at 5 and 10 years compared with the public health service, with a worse prognosis related to the advanced stages of the disease and non-white skin color. Hormone treatment contributed to the reduction of the risk of death in both services, pointing to the sustained protective effect in the private network over 10 years, most likely as a result of better guidance of the recommended treatment.

The results of this study strongly emphasize the influence of social inequality on the prognosis of breast cancer in Brazil, highlighting the need, mainly on the part of the public authorities, to reinforce strategies for BC prevention aimed at health education and communication, disease and risk factor surveillance, and early detection, in addition to guaranteeing access to the recommended treatment for all identified cases, especially in the public healthcare network.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This retrospective cohort study was approved by the appropriate ethics committee (Research Ethics Committee of the Universidade Federal de Juiz de Fora, Minas Gerais, Brazil, under reference No. 2.038.397, CAAE: 04575712.4.0000.5147), which allowed dispensing with informed consent for this research, according to national regulations (Res. CNS 196/96). The data were collected from the records of the oncology reference center, and the confidentiality of the participants was protected. We emphasize that all participants are fully anonymous, the database does not contain direct or indirect identifiers, and publication of such data does not compromise anonymity or confidentiality and does not breach local data protection laws.

Author contributions

AF, VF, JC and MG conceived the study. JC, ADC, VF, CS, CJ and MG were responsible for the data collection, analysis, and interpretation. AF, VF, MN, AC, MB-T and MG designed the analysis and wrote the manuscript. All authors (AF, MG, MB-T, VF, JC, MN, ADC, CS, AC, and CJ) commented on the initial drafts of the manuscript, critically reviewed the manuscript, and read and approved the final version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2021) 71(3):209–49. doi: 10.3322/caac.21660

2. Brazil I. *The context of breast cancer in Brazil: synthesis of data from information systems*. Rio de Janeiro: INCA (2019). Available at: https://www.inca.gov.br/sites/ufu.sti.inca.local/files//media/document//a_situacao_ca_mama_brasil_2019.pdf.

3. Goss PE, Lee BL, Badovinac-Crnjevic T, Strasser-Weippl K, Chavarri-Guerra Y, Louis JS, et al. Planning cancer control in Latin America and the Caribbean. *Lancet Oncol* (2013) 14(5):391–436. doi: 10.1016/S1470-2045(13)70048-2

4. Unger-Saldaña K, Miranda A, Zarco-Espinosa G, Mainero-Ratchelous F, Bargalló-Rocha E, Miguel Lázaro-León J. Health system delay and its effect on clinical stage of breast cancer: multicenter study. *Cancer*. (2015) 121(13):2198–206. doi: 10.1002/cncr.29331

5. Allemani C, Matsuda T, Di Carlo V, Harewood R, Matz M, Nikšić M, et al. Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): analysis of individual records for 37513025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. *Lancet*. (2018) 391(10125):1023–75. doi: 10.1016/S0140-6736(17)33326-3

6. Mendonça GAS, Silva AM, Caula WM. Tumor characteristics and five-year survival in breast cancer patients at the national cancer institute, Rio de Janeiro, Brazil. *Cad Saude Publica.* (2004) 20(5):1232–9. doi: 10.1590/S0102-311X2004000500017

7. De Moraes AB, Zanini RR, Turchiello MS, Riboldi J, De Medeiros LR. Survival study of breast cancer patients treated at the hospital of the federal university in Santa maria, Rio grande do sul, Brazil. *Cad Saude Publica.* (2006) 22(10):2219–28. doi: 10.1590/S0102-311X2006001000028

8. Schneider IJC, d'Orsi E. Five-year survival and prognostic factors in women with breast cancer in Santa catarina state, Brazil. *Cad Saude Publica.* (2009) 25(6):1285–96. doi: 10.1590/S0102-311X2009000600011

9. Guerra MR, Mendonça GAS, Bustamante-Teixeira MT, Cintra JRD, Carvalho LM, Magalhães LMPV. Five-year survival and prognostic factors in a cohort of breast cancer patients treated in juiz de fora, minas gerais state, Brazil. *Cad Saude Publica.* (2009) 25(11):2455–66. doi: 10.1590/S0102-311X2009001100015

10. Ayala ALM. Survival of women with breast cancer, of a city in the south of Brazil. *Rev Bras enferm.* (2012) 65(4):566–70. doi: 10.1590/S0034-71672012000400003

11. Fujimoto RHP, Koifman RJ, Silva IF. Survival rates of breast cancer and predictive factors: a hospital-based study from western Amazon area in Brazil. *Ciênc. saúde coletiva;* (2019) 24(1):261–73. doi: 10.1590/1413-81232018241.35422016

12. Balabram D, Turra CM, Gobbi H. Survival of patients with operable breast cancer (Stages I-III) at a Brazilian public hospital-a closer look into cause-specific mortality. *BMC Cancer.* (2013) 13(434). doi: 10.1186/1471-2407-13-434

13. Fayer VA, Guerra MR, Cintra JRD, Bustamante-Teixeira MT. Ten-year survival and prognostic factors for breast cancer in the southeast region of Brazil. *Rev Bras Epidemiol.* (2016) 19(4):766–78. doi: 10.1590/1980-5497201600040007

14. Ayala ALM, Anjos JC, Cassol GA, Höfelmann DA. Survival rate of 10 years among women with breast cancer: a historic cohort from 2000-2014. *Cien Saude Colet.* (2019) 24(4):1537–50. doi: 10.1590/1413-81232018244.16722017

15. Bustamante-Teixeira MT, Faerstein E, Latorre MR. Survival analysis techniques. *Cad Saude Publica.* (2002) 18(3):579–94. doi: 10.1590/S0102-311X2002000300003

16. Sant M, Allemani C, Capocaccia R, Hakulinen T, Aareleid T, Coebergh JW, et al. Stage at diagnosis is a key explanation of differences in breast cancer survival across Europe. *Int J Cancer.* (2003) 106(3):416–22. doi: 10.1002/ijc.11226

17. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* (2019) 69(1):7–34. doi: 10.3322/caac.21551

18. Soerjomataram I, Louwman MW, Ribot JG, Roukema JA, Coebergh JW. An overview of prognostic factors for long-term survivors of breast cancer. *Breast Cancer Res Treat* (2008) 107:309–30. doi: 10.1007/s10549-007-9556-1

19. Nogueira MC, Guerra MR, Cintra JRD, Corrêa CSL, Fayer VA, Bustamante-Teixeira MT. Racial disparity in 10-year breast cancer survival: a mediation analysis using potential responses approach. *Cad Saude Publica.* (2018) 34(9):e00211717. doi: 10.1590/0102-311x00211717

20. Zahnd WE, Sherman RL, Klonoff-Cohen H, Mclafferty SL, Farner S, Rosenblatt KA. Breast cancer staging by subtype in the lower Mississippi delta region states. *Cancer Epidemiol.* (2019) 63(2019):101624. doi: 10.1016/j.canep.2019.101624

21. Boscoe FP, Henry KA, Sherman RL, Johnson CJ. The relationship between cancer incidence, stage and poverty in the united states. *Int J Cancer.* (2016) 139 (3):607–12. doi: 10.1002/ijc.30087

22. Arnold M, Rutherford MJ, Bardot A, Ferlay J, Andersson TML, Myklebust TA, et al. Progress in cancer survival, mortality, and incidence in seven high-income countries 1995-2014 (ICBP SURVMARK-2): a population-based study. *Lancet Oncol* (2019) 20(11):1493–505. doi: 10.1016/S1470-2045(19)30456-5

23. Brito C, Portela MC, Vasconcellos MTL. Survival of breast cancer women in the state of Rio de Janeiro, southeastern Brazil. *Rev Saude Publica* (2009) 43(3):481–9. doi: 10.1590/S0034-89102009000300012

24. Edwards BK, Noone A-M, Mariotto AB, Simard EP, Boscoe FP, Henley SJ, et al. Annual report to the nation on the status of cancer, 1975-2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. *Cancer.* (2014) 120(9):1290–314. doi: 10.1002/cncr.28509

25. Guerra MR, Azevedo e silva G, Nogueira MC, Leite ICG, de Oliveira RdeVC, Cintra JRD, et al. Breast cancer survival and health iniquities. *Cad Saude Publica.* (2015) 31(8):1673–84. doi: 10.1590/0102-311X00145214

26. Giovanella L, Mendoza-Ruiz A, Pilar ACA, Rosa MC, Martins GB, Santos IS, et al. Universal health system and universal health coverage: assumptions and strategies. *Ciênc saúde coletiva.* (2018) 23(6):1763–76. doi: 10.1590/1413-81232018236.05562018

27. Supplementary National Agency of Health. *Information notebook in the health insurance: beneficiaries, providers and plans* (2019). Rio de Janeiro: ANS. Available at: http://www.ans.gov.br/images/stories/Materiais_para_pesquisa/Perfil_setor/Dados_e_indicadores_do_setor/total-cad-info-jun-2019.pdf (Accessed 15 Jan 2020).

28. Liedke PE, Finkelstein DM, Szymonifka J, Barrios CH, Chavarri-Guerra Y, Bines J, et al. Outcomes of breast cancer in Brazil related to health care coverage: a retrospective cohort study. *Cancer Epidemiol Biomarkers Prev* (2014) 23(1):126–33. doi: 10.1158/1055-9965.EPI-13-0693

29. Brazil MS. *Resident population - minas gerais* (2020). Available at: http://tabnet.datasus.gov.br/cgi/tabcgi.exe?ibge/cnv/poptmg.def (Accessed 2 Apr 2020).

30. Malachias I, Leles FAG, Pinto MAS. *Minas gerais health regionalization master plan*. Belo Horizonte: Secretaria de Estado de Saúde de Minas Gerais (2010). Available at: https://www.saude.mg.gov.br/images/noticias_e_eventos/000_2016/Livro%20Plano%20Diretor%20de%20Regionalizao%20-%20PDR-SUS-MG.pdf.

31. Brazil MS. *Ministerial ordinance no 140, 27 Feb 2014. redefines the criteria and parameters of health facilities qualified in specialized care in oncology* (2014). Available at: http://bvsms.saude.gov.br/bvs/saudelegis/sas/2014/prt0140_27_02_2014.html (Accessed 19 May 2020).

32. Brazil CNF. *National registry of deceased* (2017). Available at: https://www.falecidosnobrasil.org.br/ (Accessed 2 Sep 2017).

33. Brazil RF. *Situação cadastral do cadastro de pessoas fisicas* (2017). Available at: https://servicos.receita.fazenda.gov.br/Servicos/CPF/ConsultaSituacao/ConsultaPublica.asp (Accessed 22 Oct 2017).

34. Vasconcelos I, Hussainzada A, Berger S, Fietze E, Linke J, Siedentopf F, et al. The st. gallen surrogate classification for breast cancer subtypes successfully predicts tumor presenting features, nodal involvement, recurrence patterns and disease free survival. *Breast.* (2016) 29:181–5. doi: 10.1016/j.breast.2016.07.016

35. Joshi S, Garlapati C, Aneja R. Epigenetic determinants of racial disparity in breast cancer: looking beyond genetic alterations. *Cancers (Basel).* (2022) 14(8):1903. doi: 10.3390/cancers14081903

36. Ellis L, Canchola AJ, Spiegel D, Ladabaum U, Haile R, Gomez SL. Racial and ethnic disparities in cancer survival: the contribution of tumor, sociodemographic, institutional, and neighborhood characteristics. *J Clin Oncol* (2018) 36:25–33. doi: 10.1200/JCO.2017.74.2049

37. Jemal A, Robbins AS, Lin CC, Flanders WD, DeSantis CE, Ward EM, et al. Factors that contributed to black-white disparities in survival among nonelderly women with breast cancer between 2004 and 2013. *J Clin Oncol* (2018) 36:14–24. doi: 10.1200/JCO.2017.73.7932

38. Brazil MS. *Ministerial ordinance n°2.439/GM, 8 Dec 2005. establishing the national oncological care policy: promotion, prevention, diagnosis, rehabilitation and palliative care, to be implemented in all federal units, observing skills* Vol. Seção 1. . Brasil: Diário Oficial da República Federativa do Brasil (2005). p. 80.

39. Brazil MS. *Strategic action plan for chronic non-communicable diseases (NCDs) in Brazil -2011 to 2022* (2011). Available at: http://bvsms.saude.gov.br/bvs/publicacoes/plano_acoes_enfrent_dcnt_2011.pdf (Accessed 07 May 2020).

40. Brazil C. *Decree n°13.896, 30 Oct 2019. establishing that the examinations related to the diagnosis of malignant neoplasia be carried out within a maximum period of thirty days and to start the treatment the period of sixty days* . Available at: http://www.planalto.gov.br/ccivil_03/_ato2019-2022/2019/lei/l13896.htm (Accessed 31 May 2020).

41. Azevedo e Silva G, Bustamante-Teixeira MT, Aquino EML, Tomazelli JG, Dos-Santos-Silva I. Access to early breast cancer diagnosis in the Brazilian unified national health system: an analysis of data from the health information system. *Cad Saude Publica.* (2014) 30(7):1537–50. doi: 10.1590/0102-311X00156513

42. Rezende MCR, Koch HA, Figueiredo JA, Thuler LCS. Factors leading to delay in obtaining definitive diagnosis of suspicious lesions for breast cancer in a dedicated health unit in Rio de Janeiro. *Rev Bras Ginecol Obs.* (2009) 31(2):75–81. doi: 10.1590/S0100-72032009000200005

43. Oliveira EXG, Pinheiro RS, Melo ECP, Carvalho MS. Socioeconomic and geographic constraints to access mammography in Brazil, 2003-2008. *Cienc e Saude Coletiva* (2011) 16(9):3649–64. doi: 10.1590/S1413-81232011001000002

44. Marchi AA, Gurgel MSC. Adherence to the opportunistic mammography screening in public and private health systems. *Rev Bras Ginecol Obs.* (2010) 32 (4):191–7. doi: 10.1590/S0100-72032010000400007

45. Travassos C, Williams DR. The concept and measurement of race and their relationship to public health: a review focused on Brazil and the united states. *Cad Saúde Pública.* (2004) 20(3):660–78. doi: 10.1590/S0102-311X2004000300003

46. Qin Yu X. Socioeconomic disparities in breast cancer survival: relation to stage at diagnosis, treatment and race. *BMC Cancer.* (2009) 9(364). doi: 10.1186/1471-2407-9-364

47. Akinyemiju T, Sakhuja S, Vin-Raviv N. Racial and socio-economic disparities in breast cancer hospitalization outcomes by insurance status. *Cancer Epidemiol.* (2016) 43:63–9. doi: 10.1016/j.canep.2016.06.011

48. Cabral ALLV, Giatti L, Casale C, Cherchiglia ML. Social vulnerability and breast cancer: differentials in the interval between diagnosis and treatment of women with different sociodemographic profiles. *Cien Saude Colet.* (2019) 24(2):613–22. doi: 10.1590/1413-81232018242.31672016

49. Gonçalves H, Guerra MR, Cintra JRD, Fayer VA, Brum IV, Bustamante-Teixeira MT. Survival study of triple-negative and non–Triple-Negative breast cancer in a Brazilian cohort. *Clin Med Insights Oncol* (2018) 12:1179554918790563. doi: 10.1177/1179554918790563

50. Fayaz S, Demian GA, El-Sherify M, Eissa H, Aziz M, Abuzallouf S. Triple negative breast cancer: 10-year survival update of the applied treatment strategy in Kuwait. *Gulf J Oncol* (2019) 1(29):53–9. doi: https://pubmed.ncbi.nlm.nih.gov/30957764/

51. Oliveira EXG, Melo ECP, Pinheiro RS, Noronha CP, Carvalho MS. Access to cancer care: mapping hospital admissions and high-complexity outpatient care flows. *Case Breast Cancer* (2011) 27(2):317–26. doi: 10.1590/S0102-311X2011000200013. Cad Saude Publica.

52. Hill DA, Friend S, Lomo L, Wiggins C, Barry M, Prissbutz E, et al. Breast cancer survival, survival disparities, and guideline-based treatment. *Breast Cancer Res Treat* (2018) 170(2):405–14. doi: 10.1007/s10549-018-4761-7

53. Breast Cancer Trialists E, Group C. Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. *Lancet* (2011) 378(9804):1707–16. doi: 10.1016/S0140-6736(11)61629-2

54. Recht A, Comen EA, Fine RE, Fleming GF, Hardenbergh PH, Ho AY, et al. Postmastectomy radiotherapy: an American society of clinical oncology, American society for radiation oncology, and society of surgical oncology focused guideline update. *Ann Surg Oncol* (2017) 24(1):38–51. doi: 10.1245/s10434-016-5558-8

55. Overgaard M, Hansen PS, Overgaard J, Rose C, Andersson M, Bach F, et al. Postoperative radiotherapy in high-risk premenopausal women with breast cancer who receive adjuvant chemotherapy. *N Engl J Med* (1997) 337(14):949–55. doi: 10.1056/NEJM199710023371401

56. Peto R, Davies C, Godwin J, Gray R, Pan HC, Clarke M, et al. Comparisons between diff erent polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100 000 women in 123 randomised trials. *Lancet.* (2012) 379(9814):432–44. doi: 10.1016/S0140-6736(11)61625-5

57. Brasil - Ministério da Saúde. Instituto nacional do câncer, in: *Registros de câncer* (2021). Rio de Janeiro: INCA. Available at: https://www.inca.gov.br/numeros-de-cancer/registros-de-cancer (Accessed 18 julho 2022).

frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 21 August 2023
DOI 10.3389/fonc.2023.1116569

OPEN ACCESS

EDITED BY
Nosheen Masood,
Fatima Jinnah Women University, Pakistan

REVIEWED BY
Jyothi S Prabhu,
St. John's Research Institute, India
A. Marcell Szasz,
Semmelweis University, Hungary
Linlin Guo,
The Ohio State University, United States

*CORRESPONDENCE
Nicola Fusco
nicola.fusco@ieo.it

†These authors have contributed equally to this work

‡These authors jointly directed this work

RECEIVED 05 December 2022
ACCEPTED 26 July 2023
PUBLISHED 21 August 2023

CITATION
Sajjadi E, Venetis K, Ivanova M, Noale M, Blundo C, Di Loreto E, Scarfone G, Ferrero S, Maggi S, Veronesi P, Galimberti VE, Viale G, Peccatori FA, Fusco N and Guerini-Rocco E (2023) Immune microenvironment dynamics in breast cancer during pregnancy: impact of gestational age on tumor-infiltrating lymphocytes and prognosis. *Front. Oncol.* 13:1116569. doi: 10.3389/fonc.2023.1116569

Immune microenvironment dynamics in breast cancer during pregnancy: impact of gestational age on tumor-infiltrating lymphocytes and prognosis

Elham Sajjadi[1,2†], Konstantinos Venetis[1,2†], Mariia Ivanova[2], Marianna Noale[3], Concetta Blundo[4], Eugenia Di Loreto[5], Giovanna Scarfone[5], Stefano Ferrero[6,7], Stefania Maggi[3], Paolo Veronesi[1,8], Viviana E. Galimberti[8], Giuseppe Viale[1,2], Fedro A. Peccatori[9‡], Nicola Fusco[1,2*‡] and Elena Guerini-Rocco[1,2‡]

[1]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy, [2]Division of Pathology, IEO, European Institute of Oncology IRCCS, Milan, Italy, [3]Neuroscience Institute Aging Branch, National Research Council (CNR), Padua, Italy, [4]Breast Surgery Unit, Fondazione IRCCS Ca' Granda—Ospedale Maggiore Policlinico, Milan, Italy, [5]Gynecology Unit, Fondazione IRCCS Ca' Granda—Ospedale Maggiore Policlinico, Milan, Italy, [6]Division of Pathology, Fondazione IRCCS Ca' Granda—Ospedale Maggiore Policlinico, Milan, Italy, [7]Department of Biomedical, Surgical, and Dental Sciences, University of Milan, Milan, Italy, [8]Division of Breast Surgery, IEO, European Institute of Oncology IRCCS, Milan, Italy, [9]Fertility and Procreation Unit, Division of Gynecologic Oncology, IEO European Institute of Oncology IRCCS, Milan, Italy

Background: Breast cancer during pregnancy (PrBC) is a rare condition known for its aggressive clinical behavior. The presence of tumor-infiltrating lymphocytes (TILs) has been shown to have a significant impact on the prognosis of these patients. Despite some biological characteristics of the tumor that may differ depending on the gestational age, little is known about the dynamics of the immune landscape within the tumor microenvironment (TME) in PrBC. Therefore, in this study, our objective was to gain comprehensive insights into the relationship between gestational age at breast cancer diagnosis and the composition of the TME.

Methods: *n* = 108 PrBC were selected from our institutional registry and categorized based on the gestational age by trimester. For all cases, TILs were profiled according to the International TILs Working Group recommendations, and subtyped by CD4, CD8, and forkhead box P3 (FOXP3) immunohistochemistry. PD-L1 was tested according to the combined positive score (CPS) using the IHC 22C3 pharmDx assay, with a cutoff value of ≥10 for positivity. The statistical approach encompassed Fisher's and Chi-squared tests, with appropriate adjustments for multiple comparisons, logistic regression models, and survival analyses based on the Kaplan–Meier method.

Results: The proportion of patients with poorly differentiated (G3) neoplasms increased as the gestational age advanced (first trimester, *n* = 25, 56.8%; second trimester, *n* = 27, 69.2%; third trimester, *n* = 21, 87.5%; *p* = 0.03). The histologic

subtypes as well as the hormone receptor (HR) and HER2 status did not show significant changes across different pregnancy trimesters. In the HR+/HER2− subtype, there was a higher proportion of tumors with high/moderate TILs in the early phases of pregnancy, similar to FOXP3 expression (TILs: first trimester, n = 10, 35.7%; second trimester, n = 2, 10.5%; third trimester, n = 0; p = 0.02; FOXP3: first trimester, n = 10, 40%; second trimester, n = 3, 15.8%; third trimester, n = 0; p = 0.03). The median follow-up for our cohort was 81 months. Patients who relapsed after a breast cancer diagnosis during the first trimester were more frequently PD-L1-negative, unlike those with no disease recurrence (n = 9, 100% vs. n = 9, 56.3%; p = 0.03; hormone therapy and n = 9, 100% vs. n = 7, 53.9%; p = 0.02; chemotherapy). No statistically significant differences were seen among the three trimesters in terms of survival outcome.

Conclusion: The TME dynamics of HR+/HER2− PrBC vary based on gestational age, suggesting that immune tolerance expression during later gestational age could explain the increased aggressiveness of tumors diagnosed at that stage.

KEYWORDS

breast cancer during pregnancy, pregnancy-associated breast cancer, PD-L1, Foxp3, tumor microenvironment, breast cancer, tumor-infiltrating lymphocytes (TILs)

1 Introduction

Breast cancer is a commonly occurring malignancy during pregnancy, accounting, albeit rare, for ~4% of early-onset breast cancers (EOBCs) (1, 2). The clinical characteristics of breast cancer during pregnancy (PrBC) often manifest as advanced tumor stage, nodal involvement, and poorly differentiated histologies, indicating a more aggressive disease presentation (3, 4). Existing guidelines recommend the adoption of breast cancer standard treatment protocols for patients with PrBC (5, 6). However, it is important to consider potential modifications to these approaches due to possible delays in the diagnosis caused by physiological changes that occur during pregnancy (6–8).

There have been reports highlighting the similarities between the immunological characteristics and mechanisms at the maternal–fetal interface and those observed in tumors (9, 10). These similarities include the mechanisms involved in maternal–fetal tolerance and tumor-host immunoediting (11). Regulatory T cells (Tregs) and programmed death-ligand 1 (PD-L1) play pivotal roles in embryo implantation and induction of maternal–fetal tolerance during pregnancy (12–14). However, the potential impact of these changes on breast cancer development and progression remains a subject of debate (15). In our previous work, we conducted a comprehensive characterization of the tumor microenvironment (TME) in a large cohort of PrBC cases (16). Our findings revealed distinctive immunological and biological features of PrBC compared to conventional EOBC. Significant differences were observed between the two groups regarding the presence of tumor-infiltrating lymphocytes (TILs) and their subpopulations, as well as the expression of PD-L1. Patients with PrBC exhibited a significantly higher risk of relapse and mortality compared to those with EOBC, particularly among those with CD8+ TILs. However, the prevalence of TILs and their prognostic significance in PrBC remain controversial, with some studies reporting a low prevalence of TILs and others observing similar clinical outcomes to EOBC (17–24).

Several studies have examined the clinicopathological alterations and prognoses associated with breast cancer diagnosed at different gestational ages (3, 25). It has been observed that the histopathological characteristics of the tumors vary significantly across gestational trimesters. Specifically, individuals diagnosed later during pregnancy often exhibit a hormone receptor (HR)-negative phenotype and experience worse clinical outcomes (3). These findings suggest the existence of distinct biological profiles of PrBC that are influenced by gestational age. Taken together, there is a need to elucidate PrBC biological dynamics and better understand the role of the immune system in these tumors. Our objective was to offer a comprehensive understanding of the correlation between the gestational age at PrBC and the varying composition of the TME.

2 Materials and methods

2.1 Patients and tissue specimens

The patients included in this study were jointly diagnosed and treated at the European Institute of Oncology (IEO), Milan, Italy, and Fondazione IRCCS Ca` Granda – Ospedale Maggiore Policlinico, Milan, Italy, between February 2002 and November 2017. The study received ethical approval from the local Ethical Committees under protocol numbers #620_2018bis and #UID3472.

From our datasets, we retrieved all patients with PrBC and categorized them based on the trimester in which they were diagnosed. Representative formalin-fixed, paraffin-embedded (FFPE) blocks were carefully selected to construct tissue microarrays (TMAs) for subsequent analyses. Specifically, we generated four TMAs, each containing 180 tumor cores, resulting in a total of 720 tissue spots (with an average of 6.9 tumor samples per patient; range, 5–7 samples). For each case, the TMA sampling included both the core and periphery (i.e., invasive front) of the tumor, as well as matched normal epithelial breast tissue (i.e., glandular tissue with at least one non-neoplastic terminal ductal-lobular unit adjacent to the tumor). Our TMA protocol was optimized for immunohistochemistry (IHC) studies targeting intratumor heterogeneity in FFPE archival tissue blocks of breast cancers (26). Each case underwent thorough review, reclassification, and regrading based on the latest World Health Organization (WHO) classification of breast tumors (27) and the Nottingham histologic grading system (28). Pathologic restaging was performed following the 8th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (29). The molecular subtypes of breast cancer were determined based on the status of ER, PgR, Ki67, and HER2, following the recommendations of the St. Gallen International Expert Consensus (30).

2.2 Tumor-infiltrating lymphocyte analysis

TIL levels were assessed on 4-µm-thick hematoxylin and eosin-stained full-face sections at a ×200 magnification based on the recommendations of the International TILs Working Group (31). TIL percentage was reported only for the stromal compartment as the area of stromal tissue occupied by mononuclear inflammatory cells (including lymphocytes and plasma cells) over the total intratumoral stromal area. TILs outside of the tumor border and around ductal carcinoma *in situ* and normal terminal duct-lobular units were not counted. TIL percentage was recorded both as a continuous value and as sub-categories [i.e., negative (<1%), low (1%–20%), moderate (21%–50%), and high (>50%)].

2.3 Immunohistochemical analysis

The HR [i.e., estrogen receptor (ER) and progesterone receptor (PgR)], Ki67, and HER2 status were updated according to the latest breast biomarker reporting guidelines v1.5.0.1 published by the College of American Pathologists in March 2023 (available at: https://www.cap.org/protocols-and-guidelines/cancer-reporting-tools/cancer-protocol-templates, accessed 20 May 2023). HER2-low and ER-low tumors were identified using the established methodologies comprehensively described in previous studies (32–34). Subsequently, lymphocyte subtyping was performed by IHC using antibodies against CD4, CD8, and forkhead box P3 (FOXP3) on a Dako Omnis automated staining platform (Agilent, Santa Clara, CA, USA), as previously described (35–37). The presence and relative proportions of CD4−, CD8−, and FOXP3+ cells within the TME were evaluated as the percentage of positive TILs (31, 38). Then, CD4 and FOXP3 were recorded as dichotomous variables based on the cutoff value of 1%. CD8 was categorized as negative (<1%), low (1%–30%), moderate (31%–50%), and high (>50%). PD-L1 was tested according to the combined positive score (CPS) using the IHC 22C3 pharmDx assay on a Dako Link 48 platform (Agilent, Santa Clara, CA, USA), with a cutoff value of ≥10 for positivity (39–41). For each run, both positive and negative controls were included. Necrotic areas, as well as intraductal components, were excluded from the analysis. The methods and scoring systems employed are detailed in Supplementary Table S1.

2.4 Statistical analysis

Categorical variables were summarized as counts and percentages, while for continuous variables, means and standard deviations (SD) or median and Quartile 1 (Q1), Quartile 3 (Q3) were used. Normal distributions of continuous variables were tested using the Shapiro–Wilk test. Differences in the baseline characteristics between trimesters were assessed using Fisher's exact or Chi-squared tests, and Wilcoxon rank-sum test or generalized linear models after testing for homoscedasticity (Levene test), for categorical and continuous variables, respectively. Likewise, the differences between patients who experienced progression and patients who did not, and between patients who died during follow-up and patients alive at the end of the follow-up were analyzed. The association with cancer progression or death during the follow-up was analyzed by survival analysis according to the Kaplan–Meier method and the log-rank test. Two-tailed p-values <0.05 were considered statistically significant. The analyses were performed using SAS statistical package, version 9.4 (SAS Institute Inc., Cary, NC).

3 Results

3.1 Correlation between gestational age and PrBC clinicopathological features

A total of 108 women with PrBC were included in this study (age range, 22–44 years; follow-up time, 1–247 months; median time, 81 months). The majority of the patients were diagnosed during the first trimester (n = 44, 40.7%) followed by the second trimester (n = 39, 36.1%) and the third trimester (n = 25, 23.2%). The demographic and clinicopathologic characteristics of patients in each trimester of pregnancy are provided in Table 1, while the heatmap in Figure 1 presents a detailed individual-level analysis of these characteristics. The proportion of patients with high histologic grades (G3) demonstrated a significant increase with advancing gestational age (first trimester: n = 25, 56.8%; second trimester: n = 27, 69.2%; third trimester: n = 21, 87.5%; p = 0.03). This observation was accompanied by a lower proportion of patients with low tumor stage (T1) in the later periods (first trimester: n = 25, 56.8%; second trimester: n = 15, 38.5%; third trimester: n = 6, 24.0%; p = 0.02), suggesting a more aggressive tumor behavior associated with

TABLE 1 Clinicopathological characteristics of the patients included in the study, categorized according to the respective pregnancy trimester.

	First trimester *n* = 44	Second trimester *n* = 39	Third trimester *n* = 25	*p-value*
Age at diagnosis, year				0.139
Mean ± SD	34.5 ± 4.7	35.2 ± 3.7	36.6 ± 3.9	
Min, max	22, 44	29, 41	27, 43	
Histological type, *n* (%)				0.759
NST (ductal)	42 (95.5)	37 (94.5)	23 (92.0)	
Other	2 (4.5)	2 (5.1)		
LVI, *n* (%)	20 (45.5)	19 (48.7)	13 (52.0)	0.869
T, *n* (%)				
T1	25 (56.8)	15 (38.5)	6 (24.0)	
T2	14 (31.8)	16 (41.0)	17 (68.0)	
T3/4	5 (11.4)	8 (20.5)	2 (8.0)	
N+, *n* (%)	20 (45.5)	22 (56.4)	13 (52.0)	0.604
M+, *n* (%)	1 (2.3)	2 (5.6)	2 (8.0)	0.619
G3 histology, *n* (%)	25 (56.8)	27 (69.2)	21 (87.5)	0.034*
ER+, *n* (%)	2 (4.6)	4 (10.3)	3 (12.0)	0.243
Low	30 (68.2)	19 (48.7)	11 (44.0)	
Positive				
PgR+, *n* (%)	29 (65.9)	19 (48.7)	11 (44.0)	0.139
Ki67-high, *n* (%)	29 (65.9)	30 (76.9)	21 (84.0)	0.226
HER2+, *n* (%)				0.468
Low	14 (31.8)	9 (23.0)	6 (24.0)	
Positive	3 (6.8)	7 (18.0)	4 (16.0)	
Subtypes, *n* (%)				0.385
HR+/HER2–	28 (63.6)	19 (48.7)	11 (44.0)	
HER2+	3 (6.8)	7 (18.0)	4 (16.0)	
HR-/HER2–	13 (29.6)	13 (33.3)	10 (40.0)	

SD, standard deviation; NST, no special type; LVI, lymph vascular invasion; ER, estrogen receptor; PgR, progesterone receptor. Significant associations ($p < 0.05$) are highlighted with an asterisk (*).

increased gestational age. However, there were no significant changes in breast cancer subtypes or the HR and HER2 status according to the trimester of pregnancy (Table 1). Additionally, no statistically significant associations were found when comparing the prevalence of high histologic grade (G3) with breast cancer subtypes in patients diagnosed with PrBC in the last trimester (Supplementary Table S2).

3.2 Gestational age-dependent variations in the tumor microenvironment of PrBC

The analysis of TME dynamics across trimesters in the overall PrBC population did not reveal any statistically significant differences (Supplementary Table S3). However, when examining the tumor subtypes, we observed distinct patterns in HR+/HER2– breast cancers. In this subgroup of PrBC, the proportion of tumors with high/moderate TILs was significantly higher in the early phases of pregnancy compared to the later phases (first trimester: $n = 10$, 35.7%; second trimester: $n = 2$, 10.5%; third trimester: $n = 0$; $p = 0.02$). This finding corresponded to a higher proportion of patients with FOXP3+ TILs in the first months, which progressively decreased (first trimester: $n = 10$, 40%; second trimester: $n = 3$, 15.8%; third trimester: $n = 0$; $p = 0.03$), as shown in Table 2 and Figure 2. These findings highlight the dynamic changes in the TME of PrBC, specifically in HR+/HER2– tumors, with variations in immune composition based on gestational age.

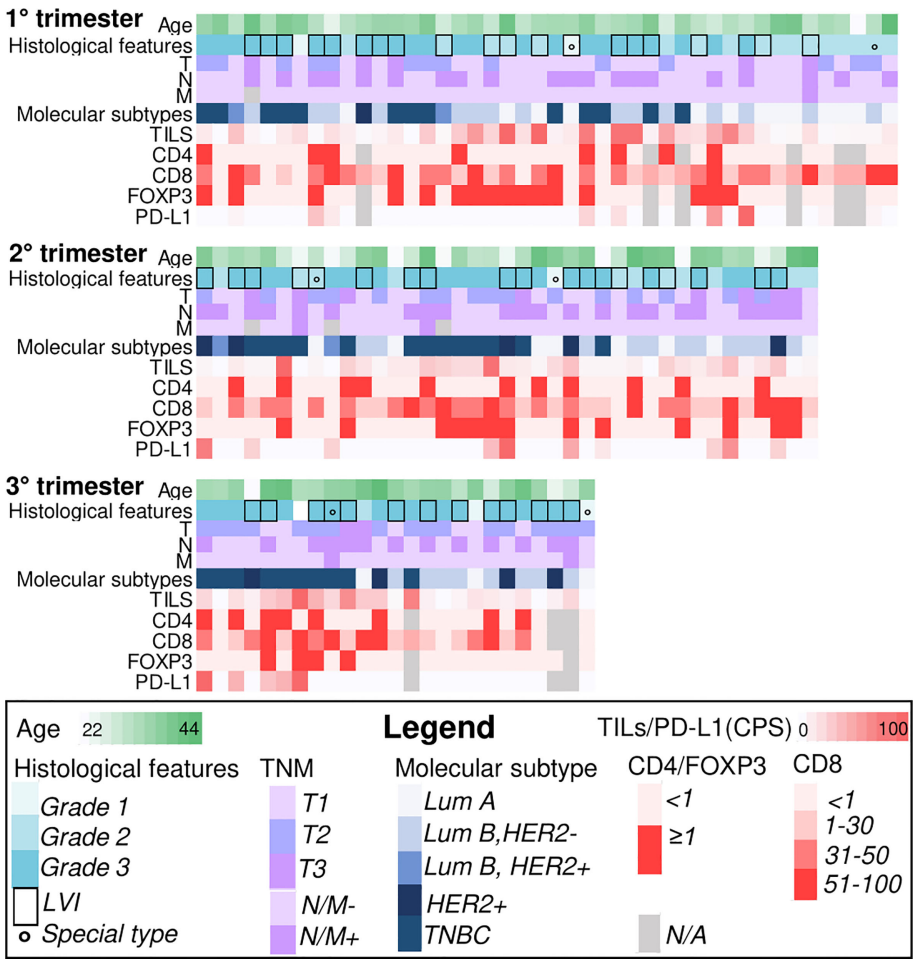


FIGURE 1
Heatmaps illustrating selected clinicopathologic and immune-related features of breast cancers during pregnancy (PrBC) categorized by trimester. Each column represents a patient, and each row represents a specific parameter, color-coded according to the legend below. TILs, tumor-infiltrating lymphocytes; FOXP3, forkhead box P3; PD-L1, programmed death-ligand 1; LVI, lymph-vascular invasion; LumA, luminal A; LumB, luminal B; TNBC, triple-negative breast cancer; N/A, not available.

3.3 Loss of PD-L1 expression as a potential indicator of disease recurrence in early pregnancy

The incidence of disease recurrence and death did not show significant differences among the trimesters, as detailed in Supplementary Figure S1, Supplementary Table S4, and Supplementary Table S5. Additionally, a higher proportion of patients who experienced disease recurrence or death showed a lack or low presence of TILs, as well as the absence of FOXP3+ and CD4+ cells. In contrast, the presence of CD8+ TILs was predominantly observed in patients with worse clinical outcomes, although statistical significance was not reached. These observations were confirmed after stratification for PD-L1 status. However, when considering score = 1 as a cutoff value for CPS, a higher proportion of patients with PD-L1 negative tumors experienced disease recurrence compared to those with CPS ≥ 1. This trend was observed across all trimesters, but statistical significance was limited to the first trimester in both endocrine therapy and chemotherapy groups (n = 9, 100% vs. n = 9, 56.3%; p = 0.03 and n = 9, 100% vs. n = 7, 53.9%; p = 0.02, respectively), as shown in Tables 3, 4. Similarly, a higher frequency of deceased patients had a lack of PD-L1 expression compared to those who survived during the follow-up period, although statistical significance was not reached (as indicated in Supplementary Table S6 and Supplementary Table S7). These results suggest that the progression of PrBC may be influenced by PD-L1 expression, observed during the early stages of pregnancy.

4 Discussion

In this study, we characterized the PrBC immune landscape dynamics based on gestational age and demonstrated that the anti-tumor immune response varies throughout pregnancy. Our study unveiled diverse immunological patterns across trimesters, linked to distinct clinical outcomes. With increasing gestational age, tumor behavior became more aggressive. TIL composition varied notably throughout trimesters, with a higher proportion of tumors having high/moderate TILs and FOXP3+ cells in early pregnancy,

TABLE 2 Distribution of tumor-infiltrating lymphocyte (TIL) subpopulations and PD-L1 expression across pregnancy trimesters, categorized by breast cancer subtypes.

	HR+/HER2− *n* = 58				HR-/HER2− *n* = 36				HER2+ *n* = 14			
	First trimester *n* = 28	Second trimester *n* = 19	Third trimester *n* = 11	*p*-value	First trimester *n* = 13	Second trimester *n* = 13	Third trimester *n* = 10	*p*-value	First trimester *n* = 3	Second trimester *n* = 7	Third trimester *n* = 4	*p*-value
TILs, *n* (%)				0.022*				0.245				0.539
≤20%	18 (64.3)	17 (89.5)	11 (100.0)		11 (84.6)	8 (61.5)	5 (50.0)		2 (66.7)	5 (71.4)	4 (100.0)	
>20%	10 (35.7)	2 (10.5)	0 (0.0)		2 (15.4)	5 (38.5)	5 (50.0)		1 (33.3)	2 (28.6)	0 (0.0)	
PD-L1 (CPS), *n* (%)				0.668				0.068				–
<10	23 (92.0)	19 (100.0)	10 (100.0)		11 (100.0)	13 (100.0)	7 (77.8)		2 (100.0)	7 (100.0)	3 (100.0)	
≥10	2 (8.0)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	2 (22.2)		0 (0.0)	0 (0.0)	0 (0.0)	
PD-L1 (CPS), *n* (%)				0.668				0.068				–
<10	23 (92.0)	19 (100.0)	10 (100.0)		11 (100.0)	13 (100.0)	7 (77.8)		2 (100.0)	7 (100.0)	3 (100.0)	
≥10	2 (8.0)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	2 (22.2)		0 (0.0)	0 (0.0)	0 (0.0)	
FOXP3, *n* (%)				0.028*				1.000				0.276
<1%	15 (60.0)	16 (84.2)	10 (100.0)		6 (54.6)	6 (46.2)	5 (55.6)		1 (50.0)	4 (57.1)	4 (100.0)	
≥1%	10 (40.0)	3 (15.8)	0 (0.0)		5 (45.4)	7 (53.8)	4 (44.4)		1 (50.0)	3 (42.9)	0 (0.0)	
CD4, *n* (%)				1.000				0.194				0.746
<1%	19 (76.0)	14 (73.7)	8 (80.0)		9 (81.8)	10 (76.9)	4 (44.4)		2 (100.0)	4 (57.1)	2 (66.7)	
≥1%	6 (24.0)	5 (26.3)	2 (20.0)		2 (18.2)	3 (23.1)	5 (55.6)		0 (0.0)	3 (42.9)	1 (33.3)	
CD8, *n* (%)				0.224				0.075				1.000
<1%	2 (8.0)	2 (10.5)	3 (30.0)		5 (45.5)	1 (7.7)	7 (11.1)		0 (0.0)	2 (28.6)	0 (0.0)	
≥1%	23 (92.0)	2 (10.5)	7 (70.0)		6 (55.5)	12 (92.3)	8 (88.9)		2 (100.0)	5 (71.4)	3 (100.0)	

HR, hormone receptors; FOXP3, forkhead box P3; PD-L1, programmed death-ligand 1; CPS, combined positive score. Significant associations ($p < 0.05$) are highlighted with an asterisk (*).

gradually declining over time. Notably, low PD-L1 expression was associated with first-trimester disease relapse.

Certain clinicopathologic characteristics in PrBC, such as advanced stages at diagnosis, high grade, and increased lymph node involvement, can vary throughout each trimester of pregnancy (42–45). It has been previously observed that breast tumors in the later stages of pregnancy are significantly more frequently of a higher grade compared to those in the first trimester (3, 6, 25). Consistent with this, our findings showed an increased prevalence of poorly differentiated neoplasms with

TABLE 3 Disease progression based on the tumor immune characteristics in patients treated with endocrine therapy.

	Disease recurrence after endocrine treatment (*n* = 64)								
	First trimester			Second trimester			Third trimester		
	No (*n* = 19)	Yes (*n* = 9)	*p*-value	No (*n* = 13)	Yes (*n* = 10)	*p*-value	No (*n* = 9)	Yes (*n* = 4)	*p*-value
TILs, *n* (%)			1.000			1.000			0.308
≤20%	12 (63.2)	6 (66.7)		12 (92.3)	9 (90.0)		9 (100.0)	3 (75.0)	
>20%	7 (36.8)	3 (33.3)		1 (7.7)	1 (10.0)		0 (0.0)	1 (25.0)	
PD-L1(CPS), *n* (%)			0.027*			1.000			1.000

(Continued)

TABLE 3 Continued

	Disease recurrence after endocrine treatment (*n* = 64)								
	First trimester			Second trimester			Third trimester		
	No (*n* = 19)	Yes (*n* = 9)	*p*-value	No (*n* = 13)	Yes (*n* = 10)	*p*-value	No (*n* = 9)	Yes (*n* = 4)	*p*-value
<1	9 (56.3)	9 (100.0)		11 (84.6)	9 (90.0)		7 (87.5)	4 (100.0)	
≥1	7 (43.8)	0 (0.0)		2 (15.4)	1 (10.0)		1 (12.5)	0 (0.0)	
FOXP3, *n* (%)			1.000			1.000			0.333
<1%	8 (50.0)	5 (55.6)		11 (84.6)	8 (80.0)		8 (100.0)	3 (75.0)	
≥1%	8 (50.0)	4 (44.4)		2 (15.4)	2 (20.0)		0 (0.0)	1 (25.0)	
CD4, *n* (%)			0.364			0.339			0.548
<1%	11 (68.8)	8 (88.9)		9 (69.2)	9 (90.0)		6 (75.0)	2 (50.0)	
≥1%	5 (31.3)	1 (11.1)		4 (30.8)	1 (10.0)		2 (25.0)	2 (50.0)	
CD8, *n* (%)			1.000			0.281			0.491
<1%	2 (12.5)	1 (11.1)		1 (7.7)	3 (30.0)		3 (37.5)	0 (0.0)	
≥1%	14 (87.5)	8 (88.9)		12 (92.3)	7 (70.0)		5 (62.5)	4 (100.0)	

TILs, tumor-infiltrating lymphocytes; FOXP3, forkhead box P3; PD-L1, programmed death-ligand 1; CPS, combined positive score. Significant associations ($p < 0.05$) are highlighted with an asterisk (*).

advancing gestational age. Not surprisingly, a lower proportion of patients had stage I tumors in later pregnancy phases, confirming the relationship between advanced gestational age and breast cancer aggressiveness. We also observed a non-significant but more pronounced proportion of Ki67 high score, LVI, and nodal involvement in the third trimester. Taken together, these findings suggest more aggressive tumor biology and provide the potential rationale for adjusting the management of high-risk individuals.

When considering the fetus as a graft, it is intriguing to contemplate the deliberate and regulated response of the maternal immune system, which has implications for both the TME and the host's overall immune capabilities. Evaluating different breast cancer subtypes, we found that TILs were higher in early pregnancy but decreased as gestation progressed in HR+/HER2– PrBC, suggesting a progressive increase in tumor immune tolerance. High TILs in breast cancer are linked to better long-term outcomes (46–48). Consistent with this, our findings revealed that patients with worse clinical behavior were more common in the last trimester, where tumors with high/moderate TILs were less frequently observed. FOXP3+ TILs were higher in early pregnancy, gradually decreasing, confirming their role in establishing immune tolerance (16, 49). Analyzing patient survival, we discovered that all cases of disease recurrence in the first trimester were PD-L1 negative, irrespective of therapy, indicating the impact of TME dynamics, like PD-L1 expression, in early pregnancy on PrBC outcomes. These results suggest that the immune response within HR+/HER2– breast cancers varies throughout pregnancy, with a higher presence of TILs and FOXP3+ TILs in the early stages. This may indicate a more active immune response against the tumor during the initial months, potentially contributing to better clinical outcomes. As the pregnancy progresses, the proportion of tumors with high/moderate TILs and FOXP3+ TILs decrease, suggesting a potential shift in the immune landscape and immune tolerance mechanisms. During pregnancy, the maternal host undergoes adaptive changes in the immune system to protect the semi-allogenic fetoplacental unit, involving attenuation of adaptive immunity and protection from innate immune defense mechanisms (50). Malignant cells can modify metabolism and signaling pathways in the TME to enhance their survival (51). These modifications can occur through various mechanisms, including the regulation of Tregs (52). Tregs play a role in immunological tolerance and can contribute to tumor immune evasion by suppressing immune responses against cancer cells. By modulating the TME and influencing the activity of Tregs, malignant cells can create an environment that supports their survival and growth. Treg cells increase during early pregnancy, likely due to their role in implantation and placental invasion of maternal tissues (49, 53, 54). Upregulated PD-L1 expression in breast cancer contributes to immunosuppression by binding to PD-1 and suppressing T-cell response (55–57). Our findings highlight the importance of tailored clinical management based on trimester and immunological profile in PrBC.

This study has limitations that should be acknowledged. Firstly, using TMAs to assess biomarker expression may not fully capture intratumor heterogeneity. To address this, we performed re-analysis on corresponding full-face sections when heterogeneity was observed. Additionally, the small sample size and potential confounding factors may impact the clinical significance of our results, particularly for HER2+ and TNBC. Further multicentric studies are needed to gain a comprehensive understanding in these subgroups. The use of a limited IHC panel with only four immune biomarkers is another inherent limitation. Expanding the

TABLE 4 Disease progression based on the tumor immune characteristics in patients treated with chemotherapy.

	Disease recurrence after chemotherapy (*n* = 82)								
	First trimester			Second trimester			Third trimester		
	No (*n* = 16)	Yes (*n* = 12)	*p*-value	No (*n* = 17)	Yes (*n* = 15)	*p*-value	No (*n* = 13)	Yes (*n* = 9)	*p*-value
TILs, *n* (%)			1.000			0.229			1.000
≤20%	11 (68.8)	9 (75.0)		11 (64.7)	13 (86.7)		10 (76.9)	7 (77.8)	
>20%	5 (31.2)	3 (25.0)		6 (35.3)	2 (13.3)		3 (23.1)	2 (22.2)	
PD-L1(CPS), *n* (%)			0.019*			1.000			1.000
<1	7 (53.9)	10 (100.0)		12 (70.6)	11 (73.3)		7 (70.0)	7 (77.8)	
≥1	6 (46.2)	0 (0.0)		5 (29.4)	4 (26.7)		3 (30.0)	2 (22.2)	
FOXP3, *n* (%)			0.680			0.131			0.285
<1%	6 (46.2)	6 (60.0)		8 (47.1)	11 (73.3)		10 (90.9)	6 (66.7)	
≥1%	7 (53.8)	4 (40.0)		9 (52.9)	4 (26.7)		1 (9.1)	3 (33.3)	
CD4, *n* (%)			0.089			0.389			1.000
<1%	7 (53.9)	9 (90.0)		10 (58.8)	11 (73.3)		6 (60.0)	5 (55.6)	
≥1%	7 (53.9)	9 (90.0)		10 (58.8)	11 (73.3)		6 (60.0)	5 (55.6)	
CD8, *n* (%)			0.604			0.319			1.000
<1%	3 (23.1)	1 (10.0)		1 (5.9)	3 (20.0)		2 (20.0)	1 (11.1)	
≥1%	10 (76.9)	9 (90.0)		1 (5.9)	3 (20.0)		2 (20.0)	1 (11.1)	

TILs, tumor-infiltrating lymphocytes; FOXP3, forkhead box P3; PD-L1, programmed death-ligand 1; CPS, combined positive score. Significant associations ($p < 0.05$) are highlighted with an asterisk (*).

examination with spatial and multiplex technologies would provide deeper insights into the immune dynamics in PrBC. Furthermore, owing to the retrospective nature of the study, comprehensive data on specific lifestyle factors were not available. Future studies should consider incorporating detailed information on lifestyle factors to enhance our understanding of PrBC. Despite these limitations, our findings offer novel insights into the TME and biology of PrBC, potentially linking to the clinical course of patients.

In conclusion, our study suggests that immune tolerance events are involved in early gestational PrBC and that decreased TILs and FOXP3 in later months may contribute to disease aggressiveness. Understanding similarities and differences between the maternal

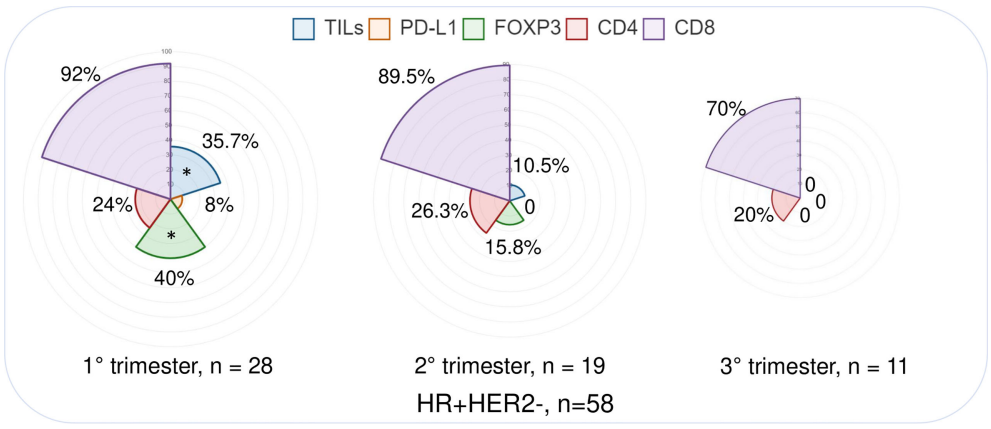


FIGURE 2
Immunograms showing the distribution of selected immune-related features in different pregnancy trimesters, focusing on the HR+/HER2– subtype. PrBC, breast cancer during pregnancy; HRs, hormone receptors; TNBC, triple-negative breast cancer; PD-L1, programmed death-ligand 1; TILs, tumor-infiltrating lymphocytes; FOXP3, forkhead box P3. Significant correlations among the different subset of patients (color-coded based on the legends) are highlighted with a star (*).

immune system and the TME provides novel insights for tailored patient management. Consideration of trimester-specific immune profiles is important for PrBC clinical decision-making. Further research is needed to uncover underlying mechanisms and their impact on outcomes.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The study was approved by the local Ethical Committees under protocol numbers #620_2018bis and #UID3472. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, FP, NF, and EG-R. Methodology, ES and NF. Histological assessment, NF. Statistical analysis, ES and MN. Resources, SF, GV, and EG-R. Data curation, ES. Writing—original draft preparation, ES, KV, and MI. Review, CB, EL, GS, SM, PV, GV, VG, FP, and EG-R. Writing and editing, ES and NF. Figures, NF and ES. Supervision, NF. All authors contributed to the article and approved the submitted version.

Funding

This research was partially funded by the Italian Ministry of Health with Ricerca Corrente 5 × 1000 funds and by the Oncomine Clinical Research Grant Program. MI was supported by Fondazione Umberto Veronesi. KV was supported by Fondazione IEO – MONZINO.

Acknowledgments

The authors acknowledge support from the University of Milan through the APC initiative.

Conflict of interest

GV has received honoraria for consulting, advisory role, and/or speaker bureau from Merck Sharp & Dohme MSD Oncology, Pfizer, Dako, Roche/Genetech, Astellas Pharma, Novartis, Bayer, Daiichi, Sankyo, Menarini, Ventana Medical Systems Dako/Agilent Technologies, Cepheid, and Celgene. NF has received honoraria for consulting, advisory role, speaker bureau, travel, and/or research grants from MSD, Boehringer Ingelheim, Novartis, AstraZeneca, Daiichi Sankyo, GlaxoSmithKline GSK, Gilead, Diaceutics, Adicet Bio, and Sermonix. FP from Merck, Ipsen, and Roche Diagnostics. EG-R from Thermo Fisher Scientific, Novartis, AstraZeneca, Roche, Biocartis, Exact Science, GSK, and Illumina. These companies had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and/or in the decision to publish the results.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1116569/full#supplementary-material

References

1. Amant F, Lefrère H, Borges VF, Cardonick E, Lambertini M, Loibl S, et al. The definition of pregnancy-associated breast cancer is outdated and should no longer be used. *Lancet Oncol* (2021) 22(6):753–4. doi: 10.1016/S1470-2045(21)00183-2

2. Korenaga TK, Tewari KS. Gynecologic cancer in pregnancy. *Gynecol Oncol* (2020) 157(3):799–809. doi: 10.1016/j.ygyno.2020.03.015

3. Suelmann BBM, Bakhuis CFJ, van Dooijeweert C, Verloop J, Zweemer R, Linn S, et al. Prognosis of pregnancy-associated breast cancer: inferior outcome in patients diagnosed during second and third gestational trimesters and lactation. *Breast Cancer Res Treat* (2022) 192(1):175–89. doi: 10.1007/s10549-021-06471-6

4. Suelmann BBM, van Dooijeweert C, van der Wall E, Linn S, van Diest PJ. Pregnancy-associated breast cancer: nationwide Dutch study confirms a discriminatory aggressive histopathologic profile. *Breast Cancer Res Treat* (2021) 186(3):699–704. doi: 10.1007/s10549-021-06130-w

5. Poggio F, Tagliamento M, Pirrone C, Soldato D, Conte B, Molinelli C, et al. Update on the management of breast cancer during pregnancy. *Cancers (Basel)* (2020) 12(12). doi: 10.3390/cancers12123616

6. Blundo C, Giroda M, Fusco N, Sajjadi E, Venetis K, Leonardi MC, et al. Early breast cancers during pregnancy treated with breast-conserving surgery in the first trimester of gestation: A feasibility study. *Front Oncol* (2021) 11:723693. doi: 10.3389/fonc.2021.723693

7. Peccatori FA, Azim HA Jr. Managing pregnancy-associated breast cancer: Is more really better? *Breast* (2016) 30:215–6. doi: 10.1016/j.breast.2016.06.006

8. Peccatori FA, Azim HA Jr., Orecchia R, Hoekstra HJ, Pavlidis N, Kesic V, et al. Cancer, pregnancy and fertility: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* (2013) 24 Suppl 6:vi160–70. doi: 10.1093/annonc/mdt199

9. Beaman KD, Jaiswal MK, Katara GK, Kulshreshta A, Pamarthy S, Ibrahim S, et al. Pregnancy is a model for tumors, not transplantation. *Am J Reprod Immunol (New York NY 1989).* (2016) 76(1):3–7.

10. Venetis K, Sajjadi E, Peccatori FA, Guerini-Rocco E, Fusco N. Immune plasticity in pregnancy-associated breast cancer tumorigenesis. *Eur J Cancer Prev* (2023). doi: 10.1097/CEJ.0000000000000803

11. Pagni F, Guerini-Rocco E, Schultheis AM, Grazia G, Rijavec E, Ghidini M, et al. Targeting immune-related biological processes in solid tumors: We do need biomarkers. *Int J Mol Sci* (2019) 20(21). doi: 10.3390/ijms20215452

12. Robertson SA, Care AS, Moldenhauer LM. Regulatory T cells in embryo implantation and the immune response to pregnancy. *J Clin Invest.* (2018) 128 (10):4224–35. doi: 10.1172/JCI122182

13. Miko E, Meggyes M, Doba K, Barakonyi A, Szereday L. Immune checkpoint molecules in reproductive immunology. *Front Immunol* (2019) 10:846. doi: 10.3389/fimmu.2019.00846

14. Zhang Y, Ma L, Hu X, Ji J, Mor G, Liao A. The role of the PD-1/PD-L1 axis in macrophage differentiation and function during pregnancy. *Hum Reprod* (2019) 34 (1):25–36. doi: 10.1093/humrep/dey347

15. Zagouri F, Psaltopoulou T, Dimitrakakis C, Bartsch R, Dimopoulos MA. Challenges in managing breast cancer during pregnancy. *J Thorac Dis* (2013) 5 Suppl 1(Suppl 1):S62–7.

16. Sajjadi E, Venetis K, Noale M, Azim HA Jr., Blundo C, Bonizzi G, et al. Breast cancer during pregnancy as a special type of early-onset breast cancer: Analysis of the tumor immune microenvironment and risk profiles. *Cells* (2022) 11(15). doi: 10.3390/cells11152286

17. Lee GE, Mayer EL, Partridge A. Prognosis of pregnancy-associated breast cancer. *Breast Cancer Res Treat* (2017) 163(3):417–21. doi: 10.1007/s10549-017-4224-6

18. Hartman EK, Eslick GD. The prognosis of women diagnosed with breast cancer before, during and after pregnancy: a meta-analysis. *Breast Cancer Res Treat* (2016) 160 (2):347–60. doi: 10.1007/s10549-016-3989-3

19. Amant F, von Minckwitz G, Han SN, Bontenbal M, Ring AE, Giermek J, et al. Prognosis of women with primary breast cancer diagnosed during pregnancy: results from an international collaborative study. *J Clin Oncol* (2013) 31(20):2532–9. doi: 10.1200/JCO.2012.45.6335

20. Azim HA, Vingiani A, Peccatori F, Viale G, Loi S, Pruneri G. Tumour infiltrating lymphocytes (TILs) in breast cancer during pregnancy. *Breast.* (2015) 24 (3):290–3. doi: 10.1016/j.breast.2015.01.009

21. Lyons TR, Schedin PJ, Borges VF. Pregnancy and breast cancer: when they collide. *J mammary gland Biol neoplasia.* (2009) 14(2):87–98. doi: 10.1007/s10911-009-9119-7

22. Johansson AL, Andersson TM, Hsieh CC, Jirstrom K, Dickman P, Cnattingius S, et al. Stage at diagnosis and mortality in women with pregnancy-associated breast cancer (PABC). *Breast Cancer Res Treat* (2013) 139(1):183–92. doi: 10.1007/s10549-013-2522-1

23. Sajjadi E, Venetis K, Fusco N. Letter to the editor regarding the article "pregnancy-associated breast cancer: a multicenter study comparing clinicopathological factors, diagnosis, and treatment outcomes with non-pregnant patients". *Breast Cancer Res Treat* (2023) 199(3):587–8. doi: 10.1007/s10549-023-06951-x

24. Tan QT, Alcantara VS, Sultana R, Loh KW, Go AL, Wong FY. Pregnancy-associated breast cancer: a multicenter study comparing clinicopathological factors, diagnosis and treatment outcomes with non-pregnant patients. *Breast Cancer Res Treat* (2023) 198(1):53–66. doi: 10.1007/s10549-022-06855-2

25. Suelmann BBM, van Dooijeweert C, Bakhuis CFJ, Linn S, van der Wall E, van Diest PJ. Pregnancy-associated breast cancer: the influence of gestational age. *Endocr Relat Cancer.* (2022) 29(3):129–38. doi: 10.1530/ERC-21-0206

26. Ercoli G, Lopez G, Ciapponi C, Corti C, Despini L, Gambini D, et al. Building up a high-throughput screening platform to assess the heterogeneity of HER2 gene amplification in breast cancers. *J Vis Exp* (2017) 130). doi: 10.3791/56686-v

27. Hoon Tan P, Ellis I, Allison K, Brogi E, Fox SB, Lakhani S, et al. The 2019 WHO classification of tumours of the breast. *Histopathology.* (2020).

28. Rakha EA, El-Sayed ME, Lee AH, Elston CW, Grainge MJ, Hodi Z, et al. Prognostic significance of Nottingham histologic grade in invasive breast carcinoma. *J Clin Oncol* (2008) 26(19):3153–8. doi: 10.1200/JCO.2007.15.5986

29. Amin MB, Edge SB, Greene FL, Byrd DR, Brookland RK, Washington MK, et al. *AJCC cancer staging manual. Eight Edition ed.* Springer International Publishing (2017).

30. Curigliano G, Burstein HJ P, Winer E, Gnant M, Dubsky P, Loibl S, et al. De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer 2017. *Ann Oncol* (2017) 28(8):1700–12.

31. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol* (2015) 26(2):259–71. doi: 10.1093/annonc/mdu450

32. Venetis K, Crimini E, Sajjadi E, Corti C, Guerini-Rocco E, Viale G, et al. HER2 low, ultra-low, and novel complementary biomarkers: Expanding the spectrum of HER2 positivity in breast cancer. *Front Mol Biosci* (2022) 9:834651. doi: 10.3389/fmolb.2022.834651

33. Fusco N, Ragazzi M, Sajjadi E, Venetis K, Piciotti R, Morganti S, et al. Assessment of estrogen receptor low positive status in breast cancer: Implications for pathologists and oncologists. *Histol Histopathol.* (2021) 36(12):1235–45.

34. Sajjadi E, Guerini-Rocco E, De Camilli E, Pala O, Mazzarol G, Venetis K, et al. Pathological identification of HER2-low breast cancer: Tips, tricks, and troubleshooting for the optimal test. *Front Mol Biosci* (2023) 10:1176309. doi: 10.3389/fmolb.2023.1176309

35. Fusco N, Lopez G, Corti C, Pesenti C, Colapietro P, Ercoli G, et al Mismatch Repair Protein Loss as a Prognostic and Predictive Biomarker in Breast Cancers Regardless of Microsatellite Instability. *JNCI Cancer Spectr.* (2018) 2(4):pky056. doi: 10.1093/jncics/pky056.

36. Fusco N, Guerini-Rocco E, Augello C, Terrasi A, Ercoli G, Fumagalli C, et al. Recurrent NAB2-STAT6 gene fusions and oestrogen receptor-α expression in pulmonary adenofibromas. *Histopathology.* (2017) 70(6):906–17. doi: 10.1111/his.13165

37. Fusco N, Vaira V, Righi I, Sajjadi E, Venetis K, Lopez G, et al. Characterization of the immune microenvironment in Malignant pleural mesothelioma reveals prognostic subgroups of patients. *Lung Cancer.* (2020) 150:53–61. doi: 10.1016/j.lungcan.2020.09.026

38. Dieci MV, Radosevic-Robin N, Fineberg S, van den Eynden G, Ternes N, Penault-Llorca F, et al. Update on tumor-infiltrating lymphocytes (TILs) in breast cancer, including recommendations to assess TILs in residual disease after neoadjuvant therapy and in carcinoma in situ: A report of the International Immuno-Oncology Biomarker Working Group on Breast Cancer. *Semin Cancer Biol* (2018) 52(Pt 2):16–25. doi: 10.1016/j.semcancer.2017.10.003

39. Sajjadi E, Venetis K, Scatena C, Fusco N. Biomarkers for precision immunotherapy in the metastatic setting: hope or reality? *Ecancermedicalscience* (2020) 14:1150. doi: 10.3332/ecancer.2020.1150

40. Criscitiello C, Guerini-Rocco E, Viale G, Fumagalli C, Sajjadi E, Venetis K, et al. Immunotherapy in breast cancer patients: A focus on the use of the currently available biomarkers in oncology. *Anticancer Agents Med Chem* (2022) 22(4):787–800. doi: 10.2174/1871520621666210706144112

41. Cortes J, Cescon DW, Rugo HS, Nowecki Z, Im S-A, Yusof MM, et al. Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): a randomised, placebo-controlled, double-blind, phase 3 clinical trial. *Lancet* (2020) 396(10265):1817–28. doi: 10.1016/S0140-6736(20)32531-9

42. Beadle BM, Woodward WA, Middleton LP, Tereffe W, Strom EA, Litton JK, et al. The impact of pregnancy on breast cancer outcomes in women<or=35 years. *Cancer* (2009) 115(6):1174–84. doi: 10.1002/cncr.24165

43. Rodriguez AO, Chew H, Cress R, Xing G, McElvy S, Danielsen B, et al. Evidence of poorer survival in pregnancy-associated breast cancer. *Obstet Gynecol.* (2008) 112 (1):71–8. doi: 10.1097/AOG.0b013e31817c4ebc

44. Murphy CG, Mallam D, Stein S, Patil S, Howard J, Sklarin N, et al. Current or recent pregnancy is associated with adverse pathologic features but not impaired survival in early breast cancer. *Cancer.* (2012) 118(13):3254–9. doi: 10.1002/cncr.26654

45. Baulies S, Cusido M, Tresserra F, Fargas F, Rodriguez I, Ubeda B, et al. Biological and pathological features in pregnancy-associated breast cancer: a matched case-control study. *Eur J Gynaecol Oncol* (2015) 36(4):420–3.

46. Srivastava G, Pant L, Kudesia M, Singh S, Gupta R. Stromal microenvironment namely angiogenesis, tumor-infiltrating lymphocytes, and matrix metalloproteinase in invasive breast carcinoma: Do they have a prognostic role? *J Cancer Res Ther* (2022) 18 (6):1733–7. doi: 10.4103/jcrt.jcrt_95_21

47. Fusco N, Sajjadi E, Venetis K, Ivanova M, Andaloro S, Guerini-Rocco E, et al. Low-risk triple-negative breast cancers: Clinico-pathological and molecular features. *Crit Rev Oncol Hematol* (2022) 172:103643. doi: 10.1016/j.critrevonc.2022.103643

48. Beckers RK, Selinger CI, Vilain R, Madore J, Wilmott JS, Harvey K, et al. Programmed death ligand 1 expression in triple-negative breast cancer is associated with tumour-infiltrating lymphocytes and improved outcome. *Histopathology.* (2016) 69(1):25–34. doi: 10.1111/his.12904

49. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. *J Reprod Immunol* (2017) 124:44–53. doi: 10.1016/j.jri.2017.10.045

50. Smith-Jackson K, Harrison RA. Alternative pathway activation in pregnancy, a measured amount "complements" a successful pregnancy, too much results in adverse events. *Immunol Rev* (2022). doi: 10.1111/imr.13169

51. Aftab S, Shakoori AR. Glucose deprivation promotes cancer cell invasion by varied expression of EMT structural proteins and regulatory molecules in MDA-MB-231 triple-negative breast cancer cells. *Crit Rev Eukaryot Gene Expr.* (2022) 33(1):53–66.

52. Ander SE, Diamond MS, Coyne CB. Immune responses at the maternal-fetal interface. *Sci Immunol* (2019) 4(31). doi: 10.1126/sciimmunol.aat6114

53. Lu L, Barbi J, Pan F. The regulation of immune tolerance by FOXP3. *Nat Rev Immunol* (2017) 17(11):703–17. doi: 10.1038/nri.2017.75

54. Guerin LR, Prins JR, Robertson SA. Regulatory T-cells and immune tolerance in pregnancy: A new target for infertility treatment? *Hum Reprod Update* (2009) 15 (5):517–35. doi: 10.1093/humupd/dmp004

55. Goff SL, Danforth DN. The role of immune cells in breast tissue and immunotherapy for the treatment of breast cancer. *Clin Breast Cancer.* (2021) 21(1): e63–73. doi: 10.1016/j.clbc.2020.06.011

56. Zerdes I, Sifakis EG, Matikas A, Chrétien S, Tobin NP, Hartman J, et al. Programmed death-ligand 1 gene expression is a prognostic marker in early breast cancer and provides additional prognostic value to 21-gene and 70-gene signatures in estrogen receptor-positive disease. *Mol Oncol* (2020) 14(5):951–63. doi: 10.1002/1878-0261.12654

57. Matzinger P. Autoimmunity: Are we asking the right question? *Front Immunol* (2020) 13:864633. doi: 10.3389/fimmu.2022.864633

Frontiers in Oncology

Advances knowledge of carcinogenesis and tumor progression for better treatment and management

The third most-cited oncology journal, which highlights research in carcinogenesis and tumor progression, bridging the gap between basic research and applications to imrpove diagnosis, therapeutics and management strategies.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact