Journal of Oncology

New Insights into the Screening, Prompt Diagnosis, Management, and Prognosis of Breast Cancer

Guest Editors: Philippe-Richard J. Domeyer, Theodoros N. Sergentanis, and Flora Zagouri

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Contents

New Insights into the Screening, Prompt Diagnosis, Management, and Prognosis of Breast Cancer
Philippe-Richard J. Domeyer and Theodoros N. Sergentanis
Editorial (2 pages), Article ID 8597892, Volume 2020 (2020)

Can We Identify or Exclude Extensive Axillary Nodal Involvement in Breast Cancer Patients Preoperatively?
Martijn Leenders, Gaëlle Kramer, Kamar Belghazi, Katya Duvivier, Petrousjka van den Tol, and Hermien Schreurs
Review Article (10 pages), Article ID 8404035, Volume 2019 (2019)

Prospective Long-Term Follow-Up of Pulmonary Diffusion Capacity Reduction Caused by Dose-Dense Chemotherapy in Patients with Breast Cancer
Yosef Landman, Salomon Marcello Stemmer, Aaron Sulkes, Victoria Neiman, Tal Granot, Daniel Hendler, Mordechai Reuven Kramer, Karen Gelmon, and Rinat Yerushalmi
Research Article (7 pages), Article ID 2584859, Volume 2019 (2019)

Relationship between Mammographic Density and Age in the United Arab Emirates Population
Young-Joon Kang, Soo Kyung Ahn, Seung Ja Kim, Hyeyoung Oh, Jaihong Han, and Eunyoung Ko
Research Article (6 pages), Article ID 7351350, Volume 2019 (2019)

Polymorphisms in Survivin (BIRC5 Gene) Are Associated with Age of Onset in Breast Cancer Patients
Ilona Sušac, Petar Ozretić, Maja Gregorić, Mirela Levačić Cvok, Maja Sabol, Sonja Levanat, Diana Trnski, Domagoj Eljuga, Sven Seiwerth, Gorana Aralica, Mladen Stanec, and Vesna Musani
Research Article (10 pages), Article ID 3483192, Volume 2019 (2019)

Factors Associated with Initial Mode of Breast Cancer Detection among Black Women in the Women's Circle of Health Study
Holly A. Szukis, Bo Qin, Cathleen Y. Xing, Michelle Doose, Baichen Xu, Jennifer Tsui, Yong Lin, Kim M. Hirshfield, Christine B. Ambrosone, Kitaw Demissie, Chi-Chen Hong, Elisa V. Bandera, and Adana A. M. Llanos
Research Article (18 pages), Article ID 3529651, Volume 2019 (2019)

TP53 Mutations Promote Immunogenic Activity in Breast Cancer
Zhixian Liu, Zehang Jiang, Yingsheng Gao, Lirui Wang, Cai Chen, and Xiaosheng Wang
Research Article (19 pages), Article ID 5952836, Volume 2019 (2019)

Imaging Biomarkers as Predictors for Breast Cancer Death
Wendy Yi-Ying Wu, Laszlo Tabar, Tibor Tot, Ching-Yuan Fann, Amy Ming-Fang Yen, Sam Li-Sheng Chen, Sherry Yueh-Hsia Chiu, May Mei-Sheng Ku, Chen-Yang Hsu, Kerri R. Beckmann, Robert A. Smith, Stephen W. Duffy, and Hsiu-Hsi Chen
Research Article (12 pages), Article ID 2087983, Volume 2019 (2019)

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Editorial

New Insights into the Screening, Prompt Diagnosis, Management, and Prognosis of Breast Cancer

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Breast cancer is the most common cancer in women and the second main cause of cancer death after lung cancer. It is one of the few cancers where large-scale secondary prevention (screening) programs are of proven value. The advent of new technologies and biomarkers is currently enriching our portfolio for the prompt diagnosis of breast cancer and taking steps beyond the traditional diagnostic approaches, which have been shown to be cost-effective and are credited for the decline in mortality of breast cancer. Groups at high risk for breast cancer, such as mutation carriers, require intense and clear screening guidelines. In this special issue, we intend to cover the most recent trends regarding the aspects of screening, prompt diagnosis, management, and prognosis in the field of breast cancer.

The study by H. A. Szukis et al. evaluated factors associated with the initial mode of breast cancer detection in a sample of 1,322 Black women in the Women's Circle of Health Study. History of routine screening mammogram was associated with lower odds of clinical breast exam (CBE) as the initial mode of detection; on the other hand, lower body mass index, performance of breast self-examination before diagnosis, and larger tumor size were associated with increased odds of self-detection versus screening mammogram.

The paper by Y.-J. Kang et al. aimed to determine the relationship between breast density and age in the United Arab Emirates and consequently to assess if the results have implications on screening guidelines for breast cancer in this country, using a retrospective study design. The authors observed a significant inverse correlation between breast density category and age. Compared to Lebanese and Western women, the proportion of Emirati women with dense breasts was lower.

M. Leenders et al. undertook a review in the Cochrane Library, Embase, and PubMed databases up to June 2019 to assess whether extensive axillary nodal involvement can be preoperatively identified or excluded in breast cancer patients. After meticulous examination of the published studies, Leenders et al. portrayed the significant limitations of all current preoperative axillary imaging modalities in the identification/exclusion of extensive nodal involvement although negative PET/CT and negative MRI results are rather promising.

The cohort study by Y. Landman et al. examined carbon monoxide diffusing capacity (DLCO) in patients with early breast cancer without preexisting lung disease, who received anthracycline- and taxane-based adjuvant dose-dense chemotherapy (DDC). After implementation of longitudinal general linear models, the results highlighted a decrease in DLCO years after DDC, especially in older patients, a finding that points to a persistent symptomatic DLCO impairment in some cases, whereas the majority of patients recover partly.

The paper by W. Y.-Y. Wu et al. estimated the independent effects of the imaging biomarkers and other predictors on the risk of breast cancer death, using a prospective cohort study design. The application of imaging biomarkers along with other predictors classified twelve categories of risk for breast cancer death. In particular, it was shown that mammographic tumor appearance was an independent

predictor of risk of breast cancer death, while controlling for conventional tumor attributes and treatment modalities. Furthermore, the casting type calcifications and the architectural distortion were positively associated with breast cancer death risk.

The paper by Z. Liu et al. evaluated the immunogenic activity of TP53 mutations in promoting breast cancer. The enrichment levels of 26 immune signatures indicating activities of diverse immune cells, functions, and pathways between TP53-mutated and TP53-wildtype BCs were compared based on two large-scale BC multiomics datasets. It was found that almost all analyzed immune signatures showed significantly higher enrichment levels in TP53-mutated BCs than in TP53-wildtype BCs, and that BC immunogenicity could be enhanced by mutant p53 via regulation of p53-mediated pathways.

I. Sušac et al. evaluated the expression and polymorphisms in the *BIRC5* gene (baculoviral IAP repeat containing 5), as well as the immunohistochemical expression of the *BIRC5*-encoded protein, survivin, in Croatian women. High survivin expression significantly correlated with negative ER status and Ki-67 expression. Interestingly, the results uncovered alleles of five BIRC5 polymorphisms (–1547C > T, –644T > C, –241C > T, 9386T > C, and 9809T > C) that were associated with younger age of breast cancer onset.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

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We would like to express our gratitude to Professor F. Zagouri for her valuable contribution to this special issue. We are also grateful to all authors whose important work made this special issue possible. We hope this collection of articles will be useful to the scientific community.

Philippe-Richard J. Domeyer
Theodoros N. Sergentanis

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Review Article

Can We Identify or Exclude Extensive Axillary Nodal Involvement in Breast Cancer Patients Preoperatively?

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Background. Breast cancer treatment has rapidly changed in the last few years. Particularly, treatment of patients with axillary nodal involvement has evolved after publication of several randomized clinical trials. Omitting axillary lymph node dissection in selected early breast cancer patients with one or two positive sentinel nodes did not compromise overall survival nor regional disease control in these trials. Hence, either excluding or identifying extensive axillary nodal involvement becomes increasingly important. *Purpose*. To evaluate whether the current diagnostic modalities can accurately identify or exclude extensive axillary nodal involvement. Evaluated modalities were axillary ultrasound, ultrasound-guided needle biopsy, MRI, and PET/CT. *Methods*. A literature search was performed in the Cochrane Library, EMBASE, and PubMed databases up to June 2019. The search strategy included terms for breast cancer, lymph nodes, and the different imaging modalities. Only articles that reported pathological *N*-stage or the total number of positive axillary lymph nodes were considered for inclusion. Studies with patients undergoing neoadjuvant systemic therapy were excluded. *Conclusion*. There is no evidence that any of the current preoperative axillary imaging modalities can accurately exclude or identify breast cancer patients with extensive nodal involvement. Both negative PET/CT and negative MRI scans (with gadolinium-based contrast agents) are promising in excluding extensive nodal involvement. Larger studies should be performed to strengthen this conclusion. False-negative rates of axillary ultrasound and ultrasound-guided needle biopsy are too high to rely on negative results of these modalities in excluding extensive nodal involvement.

1. Introduction

Since the first radical mastectomy by Halsted in the late 1800s, breast cancer surgery has become much less mutilating. Currently, results of breast-conserving surgery are excellent, due to early detection by breast cancer screening programs and the application of (neo)adjuvant treatment modalities. Axillary treatment is rapidly evolving as well. The results of the ACOSOG Z0011 [1], IBCSG 23-01 [2], and AATRM 048/13/2000 [3] trials demonstrated that selected breast cancer patients with one or two positive sentinel nodes do not benefit from completion ALND (cALND) in terms of local control, disease-free survival, and overall survival [1–3]. In these trials, only clinical T1-2N0 patients undergoing breast-conserving surgery followed by whole breast irradiation and systemic therapy were included. Therefore, most national guidelines now recommend avoiding cALND in breast cancer patients with one or two positive sentinel nodes who meet these eligibility criteria. Hence, the outcome of the sentinel node biopsy (SNB) in this subgroup of patients has actually no clinical consequences. Consequently, randomized clinical trials are recently initiated to study whether SNB can be safely omitted in these patients (SOUND [4], BOOG 2013-08 [5], and INSEMA). However, the presence of extensive nodal involvement should still be excluded. Patients with extensive nodal

involvement do not meet the eligibility criteria of the ACOSOG Z0011 trial and should therefore receive axillary treatment according to the recent guidelines. Ideally, this presence of extensive nodal involvement is accurately excluded by negative results of preoperative axillary imaging modalities. Patients with extensive axillary nodal involvement are at a much higher risk for locoregional recurrence. Irradiation of regional lymph nodes is therefore advised in these patients since it improves prognosis [6–8]. Identifying extensive nodal involvement is thus an important selection factor for postoperative regional radiotherapy. Neoadjuvant systemic therapy is increasingly implemented in patients with node-positive disease. Hence, the number of positive nodes is preferably estimated by a noninvasive axillary imaging modality before the start of neoadjuvant systemic therapy.

The first aim of this review was to evaluate whether negative results of the current preoperative axillary imaging modalities can exclude extensive nodal involvement in newly diagnosed breast cancer patients. Modalities that were evaluated are axillary ultrasound (AxUS), ultrasound-guided needle biopsy (UNB), magnetic resonance imaging (MRI), and fluorine-18-fluoro-2-deoxy-D-glucose positron emission tomography/computed tomography (PET/CT). The second aim of this review was to evaluate whether these diagnostic modalities can distinguish breast cancer patients with extensive axillary nodal involvement from patients with no or limited nodal involvement.

2. Methods

A literature search was performed in the Cochrane Library, EMBASE, and PubMed databases up to June 2019. Filters used were English language and human studies. The search strategy included terms for breast cancer, lymph nodes, and the different diagnostic entities (AxUS, UNB, MRI, and PET/CT). The inclusion criteria were as follows: (1) newly diagnosed, histologically proven breast cancer patients, (2) the diagnostic modality was performed preoperatively, (3) node histology (based on SNB and/or ALND) was the reference standard for ascertaining presence/absence of metastatic nodes, (4) cALND was performed in case of a positive sentinel node, and (5) pathological *N*-stage or total number of positive nodes was reported. Exclusion criteria were (1) studies with patients undergoing neoadjuvant systemic therapy.

To determine whether negative results of preoperative axillary imaging modalities can exclude extensive nodal involvement, both negative predictive values (NPVs) and false-negative rates (FNRs) were calculated from the raw data of all included studies. For this purpose, NPV is defined as the proportion of patients with a negative test result that does not have extensive nodal involvement histologically (i.e., number of patients with a negative test result who did not have extensive nodal involvement histologically/number of patients with a negative test result). FNR is defined as the proportion of patients with extensive nodal involvement which yielded a negative test result (i.e., number of patients with extensive nodal involvement and a negative test result/number of patients with extensive nodal involvement). The combination of NPV and FNR was used to determine which diagnostic entity is the optimal test to exclude extensive nodal involvement.

To evaluate whether preoperative axillary imaging modalities can accurately distinguish patients with no or limited axillary nodal involvement from patients with extensive nodal involvement, we evaluated studies that were specifically designed for this purpose.

Currently, there is some ambiguity on the definition of extensive nodal involvement. The ACOSOG Z0011 trial defined extensive nodal involvement as ≥3 positive nodes. However, according to TNM classification, extensive nodal involvement is defined as ≥4 histologically positive nodes (pN2-3). In this review, we will quantify the number of positive nodes whenever needed to avoid ambiguity.

3. Results

3.1. Axillary Ultrasonography

3.1.1. Excluding Extensive Nodal Involvement. A number of studies on accuracy of AxUS described the total number of histologically positive nodes. In Table 1, all studies that included at least 200 patients are summarized. In studies that defined extensive nodal involvement as pN2-3 (that is ≥ 4 positive nodes), the FNR ranges from 10 to 50% (mean 18%). Thus, on average, 18% of patients with extensive nodal involvement had a normal AxUS. The NPV of a normal AxUS to exclude extensive nodal involvement ranges from 92 to 97% (mean 96%) in these studies [9–15]. In studies that defined extensive nodal involvement as ≥3 positive nodes, the FNR ranges from 30 to 37% (mean 34%). NPV in these studies ranges from 92 to 98% (mean 95%) (Table 1) [16–23].

3.1.2. Identifying Patients with Extensive Nodal Involvement. In the post-ACOSOG Z0011 era, several studies examined whether AxUS could be used to distinguish between limited and extensive axillary nodal involvement. Most studies focused on the association between the number of sonographically suspicious nodes and the extent of (pathological) nodal disease. These studies concluded that the mean number of positive axillary nodes is significantly higher amongst patients with their positive nodes identified by AxUS than by SNB [14, 19, 24]. Further, the presence of extensive nodal involvement is significantly associated with the number of sonographically suspicious nodes in these studies [12, 17, 18, 21, 25–31]. For instance, Kim et al. [18] showed that the percentage of patients with ≥3 positive nodes was 3.1% vs. 38.5% vs. 62.5% in case of ≤1, 2, or ≥3 sonographically suspicious nodes ($P < 0.001$). In multivariate analysis, the odds ratios for ≥3 positive nodes were significantly increased when two suspicious nodes were seen (OR 6.52, 95% CI 1.36–31.28) and when more than two suspicious nodes were seen (OR 21.08, 95% CI 2.57–172.86) [18]. Farrell et al. [25] showed that the mean number of histologically positive nodes was significantly higher if more than two suspicious nodes were detected sonographically compared to two or only one (10.1 vs. 7.5 vs. 5.2) [25].

Table 1: Summary of studies on axillary ultrasonography to exclude patients with extensive nodal involvement.

Study	Year	No. of patients	No. (%) of patients with extensive nodal involvement	No. of false negatives	No. of true negatives	NPV (%)	FNR (%)
Studies in which extensive nodal involvement was defined as pN2-3							
Abe et al. [9]	2013	559	60 (10.7%)	10	368	97	17
Amonkar et al. [10]	2013	439	44 (10.0%)	10	283	97	23
Jackson et al. [11]	2015	494	48 (9.7%)	14	369	96	29
Kijima et al. [12]	2010	380	67 (17.6%)	10	238	96	15
Liu et al. [13]	2018	3944	664 (16.8%)	64	2236	97	10
Wely van et al. [14]	2013	1448	178 (12.3%)	89	1094	92	50
Zhang et al. [15]	2015	1049	207 (19.7%)	33	619	95	16
Mean						96	18
Studies in which extensive nodal involvement was defined as ≥3 positive nodes							
Barco et al. [16]	2016	1533	210 (13.7%)	64	1127	95	30
Hieken et al. [17]	2013	906	76 (8.4%)	23	620	96	30
Kim et al. [18]	2019	311	19 (6.1%)	6	238	98	32
Kramer et al. [19]	2016	2130	248 (11.6%)	91	1491	94	37
Lee et al. [20]	2013	210	38 (18.1%)	12	130	92	32
Lim et al. [21]	2019	1298	180 (13.9%)	62	950	94	34
Moorman et al. [22]	2014	1060	102 (9.6%)	37	842	96	36
Morrow et al. [23]	2018	4695	594 (12.7%)	206	3374	94	35
Mean						95	34

AxUS = axillary ultrasonography; no. of false negatives = number of patients with normal/negative AxUS but histologically extensive nodal involvement; no. of true negatives = number of patients with normal/negative AxUS and histologically no/limited nodal involvement; NPV = negative predictive value; FNR = false-negative rate.

Studies differentiating between cN0, cN1, and cN2-3 disease (based on sonographic number of suspicious axillary nodes) also showed that the percentage of patients with pN2-3 disease was significantly higher in case of cN1 or cN2-3 disease compared to cN0 disease [12, 26, 27]. In patients who were sonographically classified as cN2-3, the percentage of patients with pN2-3 disease was very high, that is, 100% (2 of 2) [27], 100% (2 of 2) [26], and 84% (46 of 55) [12], respectively. However, when using this threshold of cN2-3, a lot of patients with pN2-3 were missed since the FNR was 87.5% [27], 95.7% [26], and 31.3% [12], respectively. In short, the extent of nodal involvement is significantly associated with the number of sonographically suspicious nodes. The most optimal cutoff to distinguish between limited and extensive nodal involvement has not been determined yet.

Several studies examined the association between maximum cortical thickness of the most suspicious node and the extent of nodal involvement. All studies concluded that increased cortical thickness was an independent predictor of extensive nodal involvement [10, 18, 28, 32, 33]. The most optimal cutoff value to discriminate between limited and extensive nodal involvement ranges between 3.0 and 5.0 millimeters in these studies [10, 18, 28]. For instance, Lim et al. [33] used a cutoff point of maximum cortical thickness of 4.0 millimeters. When maximum cortical thickness was larger than 4.0 millimeters, PPV to predict ≥3 positive nodes was 82.5% (33/40). However, the FNR at this cutoff was 68.6% (72/105). Hence, the majority of patients with ≥3 positive nodes were missed when using this cutoff point [33].

One study used a combination of morphology of suspicious nodes and number of sonographically suspicious nodes to predict pN2-3 disease [9]. In this study, one experienced radiologist retrospectively reviewed AxUS images of 559 patients and determined whether there were any suspicious nodes. The suspicious nodes were categorized with a grade of suspicion of high (complete or near-complete absence of fatty hilum), medium (cortical thickness >4 mm or asymmetrical cortical thickening >3 mm), or low (uniform cortical thickening of 3-4 mm). They reported that pN2-3 disease was highly likely (PPV of 82%) when there were at least two lymph nodes of high suspicion. By using this cutoff, many patients with pN2-3 disease were missed because sensitivity of predicting pN2-3 disease was only 54% [9].

3.2. Ultrasound-Guided Needle Biopsy

3.2.1. Excluding Extensive Nodal Involvement. In Table 2, we listed all studies on accuracy of AxUS followed by ultrasound-guided needle biopsy of a suspicious node (AxUS/UNB) that provided data on the definite number of histologically positive nodes. Only two studies primarily performed ultrasound-guided core needle biopsy (CNB) [26, 27]. Both studies defined extensive nodal involvement as pN2-3 disease (Table 2). All studies

Table 2: Summary of studies on US-guided needle biopsy to exclude patients with extensive nodal involvement.

Study	Year	No. of patients	No. (%) of patients with extensive nodal involvement	No. of false negatives	No. of true negatives	NPV (%)	FNR (%)
Studies in which extensive nodal involvement was defined as pN2-3							
US/FNAC							
Gipponi et al. [34]	2016	400	42 (10.5%)	15	329	96	36
Wely van et al. [14]	2013	1448	178 (12.3%)	99	1154	92	56
US/CNB							
Nijnatten van et al. [27]	2016	377	16 (4.2%)	5	339	99	31
Schipper et al. [26]	2013	577	47 (8.1%)	23	499	94	49
Studies in which extensive nodal involvement was defined as ≥3 positive nodes							
US/FNAC							
Barco et al. [16]	2016	1506	200 (13.3%)	74	1223	94	37
Farrell et al. [25]	2015	322	29 (9.0%)	9	281	97	31
Hieken et al. [17]	2013	906	76 (8.4%)	34	790	96	45
Kramer et al. [19]	2016	2130	248 (11.6%)	137	1802	93	55
Wallis et al. [35]	2018	769	36 (4.7%)	21	716	97	58
Zhu et al. [28]	2016	445	84 (18.9%)	13	314	96	15
Mean						95	43

US/FNAC = axillary ultrasonography followed by fine-needle aspiration cytology of suspicious nodes; US/CNB = axillary ultrasonography followed by core needle biopsy of suspicious nodes; no. of false negatives = number of patients with normal/negative test but histologically extensive nodal involvement; no. of true negatives = number of patients with normal/negative test and histologically no/limited nodal involvement; NPV = negative predictive value; FNR = false-negative rate.

that defined extensive nodal involvement as ≥3 positive nodes performed ultrasound-guided fine-needle aspiration cytology (FNAC). In these studies, the FNR of AxUS/FNAC ranges from 15 to 58% (mean 43%). Thus, on average, 43% of patients with ≥3 positive nodes had a negative AxUS/FNAC (either AxUS− or AxUS+/FNAC−). The NPV of a negative AxUS/FNAC to exclude ≥3 positive nodes ranges from 93 to 97% (mean 95%). Hence, the chance of ≥3 positive nodes is approximately 5% in case of a negative AxUS/FNAC (Table 2) [16, 17, 19, 25, 28, 35].

3.2.2. Identifying Patients with Extensive Nodal Involvement. Many studies evaluated the role of US/UNB in selecting patients with extensive nodal disease. Most of these studies evaluated axillary tumor burden amongst different breast cancer patient subgroups (UNB-positive versus SNB-positive patients). Recently, three meta-analyses on this subject were published [36–38]. Houssami and Turner [37] estimated that, amongst patients with positive nodes, the odds ratio for high nodal disease burden (≥4 positive nodes) was 4.38 (95% CI 3.13–6.13) for a positive UNB versus a negative UNB [37]. Wely van et al. [36] compared the number of patients with histological N1 and N2-3 disease between patients with a positive UNB (UNB+) and patients with a negative UNB but positive SNB (UNB−/SNB+). The preferred method of biopsy was FNAC in about 90% of patients. Significantly more patients in the UNB+ group had pN2-3 disease than in the UNB−/SNB+ group ($P < 0.001$), that is, 56.0% (298 of 532) versus 23.8% (59 of 248) [36]. Ahmed et al. [38] compared the number of patients with ≤2 metastatic nodes and ≥3 metastatic nodes, in addition to only considering macrometastases. They concluded that significantly more patients in the UNB+ group had ≥3 metastatic nodes compared to the SNB+ group (odds ratio 5.95, 95% CI 5.80–6.11). The mean number of macrometastatically involved nodes was 2.9 (standard error 0.2) for the UNB+ and 1.6 (standard error 0.2) for the SNB+ group. Finally, cumulative probability identified that 56.8% of UNB+ patients and 21.1% of SNB+ patients had ≥3 macrometastatic nodes [38]. In short, the mean number of positive nodes is significantly higher in UNB+ patients compared to SNB-positive patients. Approximately 56% of patients with their positive nodes identified by UNB will ultimately have extensive nodal involvement.

Ideally, these UNB+ patients with extensive nodal involvement can be distinguished from UNB+ patients with limited nodal involvement preoperatively. A few recent studies addressed this topic and compared clinical, radiological, and pathological features between these two UNB+ subgroups [30, 33, 39]. Features that were significantly associated with having extensive nodal involvement at final pathology in UNB+ patients were as follows: larger tumor size [30, 39], lobular histology [30, 39], a higher grade of lymphovascular invasion [39], maximum cortical thickness of the most suspicious node larger than 4.0 millimeters [33], and having more than one suspicious lymph node on AxUS [30, 33, 39]. For UNB+ patients with both primary tumor size on imaging ≤2 centimeters and one abnormal node on AxUS, only 27% had N2-3 disease at final pathology ($P = 0.007$) [30].

3.3. Magnetic Resonance Imaging

3.3.1. Excluding Extensive Nodal Involvement. In total, six studies evaluating the accuracy of MRI provided data on the total number of (histologically) positive nodes (Table 3). All studies used a MRI system with a coil covering breast and

Table 3: Summary of studies on MRI to exclude patients with extensive nodal involvement.

Study		Year	Field strength	No. of patients	No. (%) of patients with extensive nodal involvement	No. of false negatives	No. of true negatives	NPV (%)	FNR (%)
Studies in which extensive nodal involvement was defined as pN2-3									
Unenhanced MRI (T1w/T2w)									
Nijnatten van et al. [27]	Reader 1	2016	1.5 T	377	16 (4.2%)	3	318	99	19
	Reader 2	2016	1.5 T	377	16 (4.2%)	2	297	99	13
DCE-MRI									
Hwang et al. [40]		2013	1.5 T	349	18 (5.2%)	4	272	99	22
Hyun et al. [41]		2016	3.0 T	310	12 (3.9%)	1	256	99.6	8
Studies in which extensive nodal involvement was defined as ≥3 positive nodes									
Unenhanced MRI (T1w/T2w)									
Barco et al. [16]		2016	1.5 T	1351	182 (13.5%)	100	1066	91	55
DCE-MRI									
Hieken et al. [17]		2013	1.5 T	505	52 (10.3%)	10	327	97	19
Kim et al. [18]		2019	3.0 T	256	17 (6.6%)	2	179	99	12

MRI = magnetic resonance imaging; DCE-MRI = dynamic contrast-enhanced MRI (using gadolinium-based contrast agents); No. of false negatives = number of patients with normal/negative MRI but histologically extensive nodal involvement; no. of true negatives = number of patients with normal/negative MRI and histologically no/limited nodal involvement; NPV = negative predictive value; FNR = false-negative rate.

axilla. Three studies defined extensive nodal involvement as ≥3 positive nodes. Two of these three studies performed dynamic contrast-enhanced MRI (DCE-MRI) with gadolinium-based contrast agents [17, 18]. One of these studies performed unenhanced MRI [16]. Accuracy of DCE-MRI to exclude ≥3 positive nodes seems to be better than unenhanced MRI. The FNR of DCE-MRI ranged from 12 to 19% (mean 17%) and NPV ranged from 97 to 99% (mean 98%) (Table 3) [17, 18].

3.3.2. Identifying Patients with Extensive Nodal Involvement. Only few studies examined whether MRI could be used to distinguish between limited and extensive nodal involvement. Kim et al. [18] compared clinical, radiological, and pathological features between early breast cancer patients with ≤2 positive nodes and patients with ≥3 positive nodes. They performed DCE-MRI with gadolinium-based contrast agents (field strength of 3.0 T). In multivariate analysis, a higher number of suspicious lymph nodes on MRI was significantly associated with the presence of ≥3 positive nodes on final pathology (2 suspicious nodes, OR 69.0, $P = 0.001$; ≥3 suspicious nodes, OR = 93.55, $P < 0.001$). In patients with no or only one suspicious node on MRI, the percentage of patients with ≥3 positive nodes on final pathology was 1.8% (4 of 225). In contrast, in patients with two or more suspicious nodes on MRI, the percentage of patients with ≥3 positive nodes on final pathology was 41.9% (13 of 31) ($P < 0.001$). When using this threshold of two suspicious nodes on MRI, 4 of 17 (23.5%) patients with ≥3 positive nodes on final pathology would have been missed. Cortical morphologic changes of the most suspicious node on MRI were not independently associated with ≥3 positive nodes on final pathology in the series of Kim et al. [18]. Hieken et al. [17] performed a similar study. They differentiated between no, only one, and more than one suspicious lymph node on magnetic resonance images. Images were obtained by DCE-MRI (1.5 T) using gadolinium in 505 breast cancer patients. If no, only one, or more than one suspicious node was seen on MRI, the percentage of patients with ≥3 positive nodes on final pathology was 3.0% (10 of 337), 15.1% (11 of 73), and 32.6% (31 of 95), respectively ($P = 0.008$) [17]. When using a threshold of two suspicious nodes on MRI, 32.6% of patients had ≥3 positive nodes on final pathology. At this threshold, 40.4% (21 of 52) of patients with ≥3 positive nodes on final pathology would have been missed. Next, Hyun et al. [41] performed DCE-MRI (3.0 T) using gadolinium in 310 breast cancer patients. They differentiated between cN0, cN1, and cN2-3 based on MRI. If no abnormal nodes were identified (cN0), 0.4% (1 of 257) was ultimately shown to have pN2-3 disease by histopathology. 45 cases were staged as cN1, of which 7 were pN2-3 (15.6%). Finally, 8 cases were staged as cN2-3, of which 4 were pN2-3 (50%) [41]. A similar study was performed by Nijnatten van et al. [27]. They evaluated the axillary regions of 377 breast cancer patients on unenhanced T2-weighted sequences. Their results were virtually identical to the results of Hyun et al. [41].

At last, two studies analyzed if MRI can be used to distinguish UNB+ patients with extensive nodal involvement from UNB+ patients with limited nodal involvement. Hieken et al. [17] concluded that the chance of ≥3 positive nodes was significantly higher in UNB+ patients with more than one suspicious node on MRI compared to patients with only one suspicious node on MRI (67.6% vs. 30.3%, $P = 0.005$) [17]. In a study of Pilewskie et al. [39], a trend was shown that the rate of ≥3 positive nodes was higher in UNB+ patients with more than one suspicious node on MRI compared to only one or no suspicious nodes ($P = 0.083$).

3.4. 18F-FDG PET/CT

3.4.1. Excluding Extensive Nodal Involvement. The combination of metabolic and morphologic data can be obtained with integrated 18F-FDG PET/CT systems. Only two studies that performed 18F-FDG PET/CT reported the total number of histologically positive nodes (Table 4). One of these studies defined extensive nodal involvement as ≥3 positive

Table 4: Summary of studies on 18F-FDG PET/CT to exclude patients with extensive nodal involvement.

Study	Year	No. of patients	No. (%) of patients with extensive nodal involvement	No. of false negatives	No. of true negatives	NPV (%)	FNR (%)
Study in which extensive nodal involvement was defined as pN2-3							
Hwang et al. [40]	2013	349	18 (5.2%)	5	288	98	28
Study in which extensive nodal involvement was defined as ≥3 positive nodes							
Ahn et al. [32]	2017	364	43 (11.8%)	9	250	97	21

No. of false negatives = number of patients with normal/negative PET/CT but histologically extensive nodal involvement; no. of true negatives = number of patients with normal/negative PET/CT and histologically no/limited nodal involvement; NPV = negative predictive value; FNR = false-negative rate.

nodes [32]. In this study, 21% of patients with ≥3 positive nodes had a negative (axillary) PET/CT. The NPV of a negative (axillary) PET/CT to exclude ≥3 positive nodes was 97% in this study [32]. The study that defined extensive nodal involvement as pN2-3 had similar results: FNR 28% and NPV 98% [40].

3.4.2. Identifying Patients with Extensive Nodal Involvement. Several studies were published on axillary tumor burden according to PET/CT scans in newly diagnosed breast cancer patients with *N*+ disease [6]. However, patients were treated with neoadjuvant chemotherapy in these studies. No single study on locoregional staging with PET/CT compared the number of suspicious axillary lymph nodes on PET/CT with pathological nodal burden without systemic therapy in between. Therefore, only circumstantial evidence is available to answer the question if PET/CT is helpful in predicting the extent of axillary nodal involvement.

First, the percentages of patients with extensive nodal involvement were 23.2% [40], 32.4% [32], and 64.0% [42] in three studies when PET/CT was positive for axillary nodal involvement. These newly diagnosed breast cancer patients all had clinically negative axillae.

Fuster et al. [43] performed PET/CT in sixty consecutive patients with primary breast carcinomas larger than three centimeters. Axillary lymph node metastases were confirmed in 20 of 52 patients with ALND. In these 20 patients, 84 of 315 nodes were histologically confirmed to contain metastases. On a lymph node count, PET/CT detected 24 of these 84 metastatic lymph nodes. Hence, the best lesion-based sensitivity in their study was 28.6%. Unfortunately, the authors did not differentiate between micro- and macrometastases [43]. Heusner et al. [44] performed a similar study. They analyzed FDG avidity of all lymph nodes ≥5 mm that were noted on CT. Any focus which could be mapped to a lymph node and was considered elevated above normal was rated as positive for metastasis. PET/CT results were correlated to histopathological findings of ALND. Of 61 patients, 24 had positive nodes on final histopathology (150 positive nodes in total). In 14 true-positive PET/CT scans, 60 suspicious lymph nodes were detected. When considering these 60 lymph nodes as truly positive nodes, their best lesion-based sensitivity was 41% [44]. However, no node-to-node correlation between avid nodes on PET/CT and histologically metastatic nodes was performed in these studies. Theoretically, PET-positive nodes may have been false-positive due to inflammation for instance. Thus, the true lesion-based sensitivity for 18F-FDG PET/CT may be lower than the percentages reported in these two studies. In short, 18F-FDG PET/CT highly underestimates the number of metastatic axillary lymph nodes.

4. Discussion

Treatment of clinical T1-2N0 breast cancer patients with one or two metastatic sentinel nodes has changed after publication of the ACOSOG Z0011 [1], IBCSG 23-01 [2], and AATRM 048/13/2000 [3] trials. Omitting completion ALND in these patients did not compromise overall survival nor regional disease control. Only patients who were treated by breast-conserving surgery, whole-breast irradiation, and adjuvant systemic therapy were included in these trials. Excluding extensive nodal involvement rather than detecting occult node-positive disease has become increasingly important since then. Ideally, this task is fulfilled by preoperative axillary imaging modalities. Therefore, we questioned if negative results of AxUS, AxUS/UNB, MRI, and PET/CT can exclude the presence of extensive nodal involvement in newly diagnosed breast cancer patients. In some studies that we reviewed, the number of patients with ≥3 positive nodes was described. In other studies, the number of patients with pN1 (1 to 3 positive nodes) and pN2-3 (≥4 positive nodes) was described. We were particularly interested in excluding the presence of ≥3 positive nodes. When the presence of ≥3 positive nodes can be excluded preoperatively in breast cancer patients that meet the ACOSOG Z0011 eligibility criteria, surgically staging the axilla (by SNB) is redundant in these patients.

When applied to exclude extensive nodal involvement, the ultimate axillary staging modality is featured by a NPV of 100% and a FNR of 0%. Only ALND approaches these requirements. Even SNB is featured by a false-negative rate of 9.8% in the largest randomized clinical trial comparing SNB with ALND in patients with clinically node-negative breast cancer [45]. Using SNB as

a reference standard is therefore a drawback of this review article. In the control (cALND) arm of the ACOSOG Z0011 trial, 21.0% had ≥3 positive nodes. Despite this high rate of extensive nodal involvement, there was a very low rate of axillary recurrences in the no-ALND arm (1.6% after 5 years) [1]. This discrepancy was probably due to the effects of adjuvant systemic therapy and whole-breast irradiation, which eliminated residual axillary metastases. In our opinion, the FNRs of preoperative axillary staging methods that are applied to exclude ≥3 positive nodes (in patients who meet the ACOSOG Z0011 eligibility criteria) should not exceed this rate of 21.0%.

In Table 5, NPVs and FNRs of all preoperative axillary imaging modalities to exclude ≥3 positive nodes are summarized. NPVs of all modalities to exclude ≥3 positive nodes are very high (mean NPVs of all modalities approximately 95%). These very high NPVs can (partially) be explained by the low prevalence of ≥3 positive nodes because NPV is inversely related to prevalence. Since FNR is intrinsic to a test, the FNR might be more helpful to determine which imaging modality might best be used to exclude ≥3 positive nodes.

AxUS and AxUS/UNB are the most widely studied preoperative axillary imaging modalities. FNRs of both negative AxUS and negative AxUS/FNAC (either AxUS− or AxUS+ FNAC−) were well above the arbitrarily decided maximum FNR of 21%. Thus, when relying on negative AxUS or negative AxUS/FNAC results, too many patients with ≥3 positive nodes would be missed and would not be properly treated. This could negatively influence the number of locoregional recurrences and prognosis [6–8]. AxUS/FNAC performs worse than AxUS in excluding ≥3 positive nodes. This worse FNR of AxUS/FNAC to exclude ≥3 positive nodes can be explained by the fact that the false-negative results of FNAC are added to the false-negative results of AxUS.

Evidence regarding the diagnostic performance of both MRI and PET/CT in excluding ≥3 positive nodes is very scarce. DCE-MRI with gadolinium-based contrast agents seems to perform better in excluding ≥3 positive nodes than nonenhanced MRI albeit DCE-MRI showed less promising results in a previous systematic review on the role of MRI in axillary lymph node imaging [46]. In both studies that evaluated DCE-MRI with gadolinium-based contrast agents, the FNR of a negative MRI to exclude ≥3 positive nodes was lower than 21%. The same holds for PET/CT. Only one PET/CT study met our inclusion criteria, and in this study, the FNR of a negative PET/CT to exclude ≥3 positive nodes was 20.9%. Hence, both negative PET/CT and negative DCE-MRI (with gadolinium-based contrast agents) seem promising in excluding ≥3 positive nodes. However, this conclusion is based on only one PET/CT study and two DCE-MRI studies. Before we can rely on these diagnostic modalities in excluding ≥3 positive nodes, larger studies should be performed to confirm these results.

Our second aim was to evaluate whether these preoperative axillary imaging modalities can accurately distinguish patients with extensive nodal involvement from patients with no or only limited axillary nodal involvement. In short, none of the investigated preoperative axillary imaging modalities can. A higher number of suspicious lymph nodes on AxUS and MRI are significantly associated with extensive nodal involvement. However, the most optimal cutoff (e.g., ≥2 suspicious nodes on AxUS or MRI) to distinguish between limited and extensive nodal involvement has not been determined yet. With rising cutoff values, positive predictive values to predict extensive nodal involvement will rise, but false-negative rates will rise as well (more patients with extensive nodal involvement will be missed). Therefore, the ideal cutoff values may not exist. Mathematical models (nomograms, scores, and prediction rules) combining clinical, pathological, and radiological parameters may be more useful to estimate the chance of extensive nodal involvement in patients with suspicious nodes on AxUS or MRI and in UNB+ patients. Development of these models should be subject for future studies.

Currently, either excluding or identifying extensive nodal involvement in breast cancer patients is of great importance for reasons described above. However, it could be argued that the role of axillary staging will diminish in the near future. In the NSABP B-04 trial, clinically node-negative breast cancer patients were randomized for mastectomy, mastectomy with ALND, or mastectomy with axillary radiotherapy in the 1970s. About 40% of patients in the ALND group had pathological confirmation of tumor-positive axillary lymph nodes. However, less than half of these patients (18.6%) had clinically apparent ipsilateral nodal metastases during 25 years of follow-up. None of these patients was treated with systemic therapy or axillary radiotherapy [47]. This discrepancy between axillary recurrences in the no-ALND group and the rate of nonsentinel node metastases in the ALND group was also shown in the ACOSOG Z0011 [1] and IBCSG 23-01 trials [2]. In these latter trials, systemic therapy and radiotherapy have contributed to this discrepancy since almost all patients were treated with systemic therapy and some form of radiotherapy. Even more important, disease-free survival and overall survival were not affected by omitting ALND in any of these trials. Hence, leaving positive axillary nodes unremoved might well not be that big a problem, particularly because the majority of patients are nowadays treated with adjuvant systemic therapy that is increasingly more effective. The current FNR of 31% of a negative AxUS to exclude extensive nodal involvement might therefore be acceptable in the future. The long-term results of randomized clinical trials in which SNB is omitted and axillary staging is performed exclusively by AxUS (SOUND [4], BOOG 2013-08 [5], and INSEMA) might provide us with some answers.

Table 5: Summary of accuracy of preoperative axillary imaging modalities to exclude patients with ≥3 positive nodes.

Staging modality	No. of studies	Total no. of patients	Prevalence of extensive nodal involvement (mean)	NPV (mean)	FNR (mean)
AxUS	8	12.143	6.1–18.1 (12.1)	92–98 (95)	30–37 (34)
US/FNAC	6	6.078	4.7–18.9 (11.1)	93–97 (95)	15–58 (43)
MRI	3	2.112	6.6–13.5 (11.9%)	91–99 (93)	12–55 (45)
Unenhanced MRI	1	1.351	13.5	91	55
DCE-MRI	2	761	6.6–10.3 (9.1)	97–99 (98)	12–19 (17)
PET/CT	1	364	11.8	97	21

No. = number; FNR = false-negative rate; NPV = Negative Predictive Value; AxUS = Axillary Ultrasonography; US/FNAC = AxUS followed by fine-needle aspiration cytology of suspicious node; MRI = Magnetic Resonance Imaging; DCE = Dynamic Contrast Enhanced; PET = Positron Emission Tomography; PET/CT = combined PET and Computed Tomography.

5. Conclusion

Currently, there is no preoperative axillary imaging modality that can neither identify nor exclude breast cancer patients with extensive nodal involvement accurately. Both negative PET/CT and negative DCE-MRI scans (with gadolinium-based contrast agents) are promising in excluding ≥3 positive nodes in breast cancer patients. Larger studies should be performed to strengthen this conclusion. False-negative rates of AxUS and AxUS/FNAC are too high to rely on negative results of these modalities in excluding ≥3 positive nodes. However, with increasingly more effective (neo) adjuvant therapy, these current false-negative rates might be acceptable in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Prospective Long-Term Follow-Up of Pulmonary Diffusion Capacity Reduction Caused by Dose-Dense Chemotherapy in Patients with Breast Cancer

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Background. Our previous study of pulmonary function in 34 patients with early breast cancer without preexisting lung disease showed that anthracycline- and taxane-based adjuvant dose-dense chemotherapy (DDC) caused a significant 16.4% mean reduction in carbon monoxide diffusing capacity (DLCO). The present study reports the pulmonary and oncological outcomes of these patients on long-term follow-up. *Patients and methods*. The primary endpoint was DLCO measured by the pulmonary function test (PFT) performed at a median of 27 months after DDC (range, 8–97) in 25 patients without disease recurrence. DLCO values were recorded as a percentage of predicted values according to age, height, and hemoglobin level and analyzed relative to baseline pre-DDC DLCO values. The secondary endpoints were symptoms, additional therapies, and cancer outcomes during a median of 11 years' follow-up (range, 4.4–11.4). *Results*. A longitudinal general linear model showed significant effects of time on DLCO and its trend ($F(1, 87) = 14.68$, $p < 0.001$ and $F(1, 87) = 10.26$, $p = 0.002$, respectively). Complementary descriptive analysis showed a significant recovery on the follow-up PFT (75.6% vs. 81.9%, $p = 0.002$), but it was still significantly lower than the baseline DLCO (81.9% vs. 92.0%, $p = 0.003$). Five patients (20%) still showed a >20% relative DLCO reduction from baseline. Patients with dyspnea or fatigue at later clinical follow-up had a significantly lower DLCO value on the follow-up PFT than nonsymptomatic patients (80.5% vs. 92.1%, $p = 0.02$). DLCO recovery was inversely correlated with age ($R = -0.39$, $p = 0.05$), but no significant correlation was found with the length of time until the follow-up PFT or additional therapies. There was no association of DDC-related DLCO reduction with cancer outcomes. *Conclusions*. The significant reduction in DLCO seen after DDC in patients with potentially curable breast cancer is evident years afterwards, especially in older patients. While most patients partly recover, some will have a lasting symptomatic DLCO impairment.

1. Introduction

Better therapies and screening techniques for patients with breast cancer along with the general aging of the population have increased the number of breast cancer survivors [1–3]. This has made late toxicities from cancer care an ever-growing problem [2–4]. Awareness of the potential dangers of therapy is mandated for all practitioners and patients, as the adverse effects on daily function and health-related quality of life (HRQOL) can last many years. While dyspnea

and acute pulmonary toxicity during breast cancer care have been extensively reported and studied [5–9], data on its long-term pulmonary sequelae remain sparse.

In an earlier study, we investigated the changes in pulmonary function in 34 patients with breast cancer during the administration of adjuvant dose-dense chemotherapy (DDC) [5]. We observed a significant absolute mean reduction of 16.4% in carbon monoxide diffusing capacity (DLCO), with more than half the patients (58.8%) showing a relative decrease of more than 20% from baseline. However, most of the pulmonary injury remained subclinical, and only 5 patients (14.7%) reported grade 1 dyspnea during treatment. To our knowledge, this was the first report on pulmonary function injury with the current widely used standard-of-care DDC protocol [10, 11]. As the patients were otherwise healthy and their disease was potentially curable, long-term follow-up information on their DLCO values is highly important.

The aim of the present study was to investigate the long-term pulmonary and oncological outcomes of these patients as well as the impact of patient and treatment characteristics on DLCO recovery.

2. Patients and Methods

The original study cohort consisted of 34 consecutive female patients with breast cancer receiving adjuvant DDC at a single tertiary medical center from September 2006 to April 2007 [5]. None of the patients had any known preexisting lung disease, and all had normal findings on chest X-ray film or computed tomography scan prior to therapy. The original chemotherapy protocol consisted of 4 cycles of IV doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 (AC) with growth factor support every 14 days, followed by 12 doses of weekly IV paclitaxel 80 mg/m^2(T). Pulmonary function tests (PFTs) were originally performed before DDC administration (P1), after AC (P2), and after T (P3); 4 patients did not undergo the final P3 PFT. The full DDC regimen was completed in 33 patients. The remaining patient received 3 additional cycles of AC with 5-fluorouracil instead of T; she showed no reduction in DLCO on the P2 PFT.

For the present study, all original PFTs were reviewed and reanalyzed. Pulmonary follow-up data were available for 27 patients of the original cohort who underwent another PFT (P4) after a median of 27 months from onset of DDC (range, 8–97). We excluded 2 patients from the analysis because their later PFT was performed after disease recurrence. The primary endpoint of the study was the DLCO value at P4. The secondary endpoints were symptoms, additional therapies, disease recurrence, and mortality; these data were obtained from the medical records.

2.1. Pulmonary Function Tests. All PFTs, at baseline and thereafter, were performed at the same pulmonary laboratory using the Medical Graphics Pulmonary Function System (1070-series 2, St. Paul, MN, USA). They included spirometry, lung volume, and DLCO by single-breath technique. The predicted values of the parameters were obtained from the regression equations of the European Community for Coal and Steel [12]. DLCO values were corrected for hemoglobin level and recorded as a percentage of the predicted value according to height and age at the time of testing, thereby correcting for the effects of body structure and aging. Studies have shown that owing to the high variability among patients, DLCO changes over time and interventions are better analyzed as a percentage of the baseline DLCO values [13]. In the present report, we focused on the effect of a decline in DLCO during any part of the DDC regimen on later outcomes. Therefore, the maximal DLCO reduction in the original study was recalculated continuously as the greater reduction from baseline at either P2 or P3. As intersession variability in healthy adults has been reported be as high as 9% [12] and stricter reports state that an intersession variation of at least 16% is needed to be deemed significant [14], patients were considered to have a DDC-related DLCO injury (DRDI) if their maximal DLCO reduction was more than 20%. This cutoff was also in line with the median 19.9% reduction in DLCO seen from P1 to P3. DLCO recovery in the follow-up cohort was defined continuously as the difference in percentage from baseline between the DLCO value at P4 and the lower DLCO value at either P2 or P3.

2.2. Statistical Analysis. We used individual and means graphs to show the differences that occurred in DLCO over time and between subgroups. A longitudinal general linear model with complementary descriptive statistics was employed to evaluate differences over time and between groups. In order to evaluate associations between DLCO recovery and time of follow-up, patient characteristics, and additional therapies, we used the chi-square (χ^2) test for categorical parameters and t-tests or Pearson correlation for continuous parameters, as appropriate. Associations between DRDI and oncological outcomes were evaluated with the Cox proportional hazard regression model.

3. Results

The patients' clinical and treatment characteristics are displayed in Table 1. No significant differences were observed in any of the parameters between the original and the follow-up cohorts. Specifically, the rate of DRDI was similar in the two cohorts (58.8% vs. 64%, $p = 0.31$).

The changes in DLCO values over time were analyzed using a longitudinal general linear model with time as a continuous variable. In this manner, we were able to analyze time as both a linear and quadratic covariate. The effect of both was found to be significant ($F(1, 87) = 14.68$, $p < 0.001$ and $F(1, 87) = 10.26$, $p = 0.002$, respectively). The significant quadratic effect of time indicates a significant change in the trend of DLCO, which was negative up to P3 and positive thereafter (Figure 1(a)). Complementary descriptive statistics supported this finding, showing a significant reduction in DLCO from P1 to P3 (92.0% vs. 75.6%, $p < 0.001$) and a significant recovery at P4 (75.6% vs. 81.9%, $p = 0.002$).

Table 1: Patient and treatment characteristics: original and follow-up cohorts.

Characteristic	Original cohort ($n = 34$)	Follow-up cohort ($n = 25$)	p value
Age at diagnosis (yr), mean ± std	49.6 ± 12.5	49.9 ± 11.5	0.92
Body mass index (kg/m^2), mean ± std	27.0 ± 5.2	26.7 ± 5.0	0.82
Smoker	6 (17.6)	5 (20.0)	0.55
Stage			
I	4 (11.8)	4 (16.0)	0.2
II	21 (61.8)	14 (56.0)	0.24
III	9 (26.5)	7 (28.0)	0.73
Adjuvant endocrine therapy	24 (70.6)	19 (76.0)	0.24
Adjuvant trastuzumab	7 (20.6)	5 (20.0)	0.89
Triple negative breast cancer	8 (23.6)	5 (20.0)	0.41
Radiotherapy			
Tangential lymph nodes	32 (94.1)	23 (92.0)	0.38
Tangential + regional lymph node fields	15 (44.1)	12 (48.0)	0.44
DDC-related DLCO injury	20 (58.8)	16 (64.0)	0.31
Breast cancer recurrence	11 (32.4)	6 (24.0)	0.08
Breast cancer-related death	7 (20.6)	4 (16.0)	0.27

Values are n (%) unless otherwise stated.
Abbreviations: DDC, dose-dense chemotherapy; DLCO, carbon monoxide diffusing capacity

Figure 1: (a) Individual and mean DLCO values measured at 4 time points. A longitudinal general linear model shows a significant effect of time as both a linear and a quadratic covariate ($F(1, 87) = 14.68$, $p < 0.001$ and $F(1, 87) = 10.26$, $p = 0.002$, respectively), indicating significant changes in DLCO and in its trend, i.e., a reduction during DDC until P3 and partial recovery at follow-up. (b) When patients are grouped by the presence of DRDI, the longitudinal general linear model yields a significant interaction between DRDI and both time covariates ($F(1, 85) = 7.96$, $p = 0.006$ and $F(1, 85) = 6.79$, $p = 0.011$, respectively). Therefore, in patients with DRDI, the decrease to P3 and the recovery at P4 were significant, whereas in patients without DRDI, the changes were not significant. Thin pale lines represent individual patients; thick bright lines represent mean values. *Significantly lower than P1 at $p < 0.05$. #Significantly higher than P3 at $p < 0.05$. Abbreviations: DLCO, carbon monoxide diffusing capacity; DRDI, dose-dense chemotherapy-related DLCO injury; P1, prior to DDC administration; P2, after AC; P3, after T; P4, later follow-up.

Additionally, mean DLCO at P4 was significantly lower than mean DLCO at P1 (81.9% vs. 92.0%, $p = 0.003$).

In order to identify differences between patients with and without DRDI, a similar longitudinal general linear model was employed to explore the effects of presence of DRDI and time as a linear and quadratic covariate, with an interaction term between DRDI and both time covariates (Figure 1(b)). The results showed a significant effect of DRDI ($F(1, 32) = 8.43$, $p = 0.007$) and a significant interaction between DRDI and both time covariates ($F(1, 85) = 7.96$, $p = 0.006$ and $F(1, 85) = 6.79$, $p = 0.011$, respectively). On further exploration of this interaction, we found that the effect of time on DLCO and its trend was significant in patients with DRDI ($F(1, 52) = 14.06$, $p < 0.001$) but not in patients without DRDI ($F(1, 33) = 0$, $p = 0.98$). Complementary descriptive statistics showed that

patients with DRDI had both a significant decrease at P3 (96.9% vs. 73.3%, $p < 0.001$) and a significant recovery at P4 (73.3% vs. 82.3%, $p = 0.013$), although the value at P4 was still significantly lower than that at P1 (82.3% vs. 96.9%, $p = 0.001$). Interestingly, the baseline DLCO was higher for patients who had DRDI than for patients who did not (96.9% vs. 85.0%, $p = 0.015$). Pearson correlation analysis between the maximal DLCO reduction and the baseline DLCO value yielded significant results ($r = -0.506$, $p = 0.002$).

The mean maximal DLCO reduction during DDC was 18.9%, and the mean DLCO recovery was 9.9% (Figure 2). Nevertheless, a reduction of more than 20% from baseline was still present on the follow-up PFT in 5 of the 25 patients (20%).

To evaluate the association between age and both maximal DLCO reduction and DLCO recovery, we used the Pearson correlation. As reported in the original study, we found that age was not associated with maximal DLCO reduction ($r = 0.13$, $p = 0.45$; Figure 3(a)), but it was inversely correlated with DLCO recovery ($r = -0.39$, $p = 0.05$; Figure 3(b)).

No significant correlation was found between DLCO recovery, and the length of time elapsed between DDC administration and P4 ($r = 0.14$, $p = 0.42$). However, analysis of the results recorded in the initial 3 years after DDC revealed a trend-level correlation ($r = 0.49$, $p = 0.06$). On separate analysis of the patients with DRDI (Figure 4), the correlation strengthened and became significant ($r = 0.83$, $p = 0.006$).

No additional significant associations were found between DLCO recovery and patient and treatment characteristics, including body mass index, smoking status, staging, endocrine therapy, trastuzumab treatment, and breast or chest wall tangential irradiation, with or without lymph node fields. When only the patients with DRDI were analyzed, we found that the mean DLCO value in recovery was lower in smokers than in nonsmokers (4.5% and 15.3%) and in patients who were treated with trastuzumab than in patients who were not (6.5% and 14.0%). However, neither of these differences was statistically significant ($p = 0.16$ and $p = 0.25$, respectively).

After a median follow-up of 11 years, none of the patients had received a diagnosis of chronic pulmonary disease. Five of the 25 patients (20%) reported nonspecific dyspnea and/or fatigue (while not under endocrine therapy) one year or more after DDC administration. These patients had significantly lower DLCO values at P4 relative to baseline than patients without such complaints (80.5% vs. 92.1%, $p = 0.02$). The complaints were not age-related.

Regarding oncological outcomes, the disease recurred in 11 of the original cohort of 34 patients (32.4%), of whom 7 died (20.6%). The 20 patients in the original cohort with DRDI (58.8%) appeared to be at higher risk for both these events, but the association did not reach statistical significance (Figure 5). After the follow-up PFT, second malignancies developed in 3 patients (pancreatic adenocarcinoma, cholangiocarcinoma, and uterine cancer).

4. Discussion

The present study investigated pulmonary function over time in patients with breast cancer receiving adjuvant DDC.

Figure 2: Maximal DLCO reduction during DDC and DLCO recovery at later follow-up. Abbreviations: DLCO, carbon monoxide diffusing capacity; DRDI, dose-dense chemotherapy-related DLCO injury.

To our knowledge, this is the only study that has investigated sequential PFTs with the AC-T regimen. Several other studies reported pulmonary follow-up of comparable length. In two long-term studies of PFT after breast irradiation, the results were contradictory. Erven et al. [15] and Jaén et al. [16] showed milder reductions in DLCO during radiotherapy than in our cohort (4–7.5%). Only 35% and 51% of their patients, respectively, received adjuvant chemotherapy, almost exclusively based on cyclophosphamide, methotrexate, and 5-fluorouracil. Erven et al. [15] reported a significant mean maximal decline of 9% in DLCO at 10 years compared to baseline whereas Jaén et al. [16] reported a mean maximal decline of 3% from baseline at 2 years after treatment with a relative recovery at 7 years. These differences may be explained by the fact that the radiation therapy in the cohort of Erven et al. [15] was delivered without computed tomography planning and with more extensive lymph node fields, practices that have been found to increase lung exposure to radiation [17].

The radiotherapy administered to the cohort of Jaén et al. [16] might be more in accordance to that used in our cohort, and its mild effect on DLCO might explain the absence of an association between DLCO recovery and radiotherapy parameters in the present study. In addition, the 2-year nadir in DLCO and the later recovery in the earlier study may be in line with the trend we observed of DLCO recovery in the first 3 years after DDC administration.

Bhalla et al. [6] reported a significant 12.6% mean reduction in DLCO in 150 patients with high-risk locally advanced breast cancer treated with cyclophosphamide/doxorubicin/5-fluorouracil (CAF) as induction therapy prior to either standard-dose chemotherapy with cyclophosphamide, cisplatin, and bischloroethylnitrosourea or high-dose chemotherapy with autologous bone marrow transplantation. Follow-up data beyond 2 years were available for 19 (25%) and 22 (29%) patients from each

Figure 3: Maximal DCLO reduction (a) and recovery (b) according to age at dose-dense chemotherapy onset. Abbreviations: DLCO, carbon monoxide diffusing capacity.

Figure 4: DLCO recovery according to the length of time passed from DDC onset to the follow-up PFT in patients with DRDI. Abbreviations: DDC, dose-dense chemotherapy; DLCO, carbon monoxide diffusing capacity; DRDI, DDC-related DLCO injury.

group, respectively. The DCLO values of the standard-dose group did not decline further during treatment, with a final mean decrease of 10% from the pre-CAF baseline. The high-dose group had a mean DLCO reduction of up to 60% from baseline during treatment, and most of the patients were treated with prednisone. After 2 years, the reduction averaged 22% from the pre-CAF value. The DLCO injury associated with the CAF induction protocol was further explored by Bhalla et al. using bronchoalveolar lavage analysis. The results showed an increase in alveolar cellular inflammation markers such as IL-6, IL-8, neutrophils, and lymphocytes compared to healthy volunteers [6]. This effect is thought to be attributable mainly to the cyclophosphamide, whose by-product acrolein interferes with the antioxidant system of tissues, resulting in oxidative stress, apoptosis, and necrosis. This toxicity can rarely lead to interstitial pneumonitis and fibrosis [18, 19]. If oxidative stress is the driving mechanism of pulmonary injury, these findings might also explain the presence of DRDI in the patients with a higher baseline DLCO in our cohort. Since tissue hypoxia is associated with cancer treatment resistance [20, 21], it might be associated with lesser toxicity as well.

The greater DLCO injury in our original study (18.9%) compared to the CAF induction protocol might be explained by the greater dose intensity of AC, which was delivered every 2 weeks instead of every 3 weeks. The prophylactic use of growth factor support might also have contributed to the DLCO reduction, as it has been known to exacerbate chemotherapy-related pulmonary toxicity through activation of neutrophils and a proinflammatory cytokine response [22]. In addition, our patients received paclitaxel, which may cause pulmonary toxicity such as pulmonary infiltrates and hypersensitivity reactions and, in rare cases, pneumonitis [23, 24]. The mechanism for hypersensitivity is thought to be non-IgE-mediated given that most reactions occur during the first and second doses [25]. However, the contribution of paclitaxel to the DLCO injury, which worsened after its administration, is unclear, since it was given with proper premedication and none of the patients acquired clinical symptoms of pneumonitis.

The mean DLCO recovery observed during follow-up in the present cohort (9.9%) was roughly half the mean maximal DLCO reduction (18.9%) observed in the original study; nevertheless, in 5 patients (20%), there was a reduction of more than 20% from their baseline DLCO. Although our patients apparently had a more pronounced recovery than the cohort of Bhalla et al. [6], their patients were more heavily treated afterwards, so it is hard to draw any clear conclusions from this comparison.

Among the various symptoms affecting breast cancer survivors, fatigue was reported to have the strongest impact

Figure 5: Disease-free survival and overall survival in patients with and without DRDI. Abbreviations: DRDI, dose-dense chemotherapy-related carbon monoxide diffusing capacity injury; DFS, disease-free survival; OS, overall survival.

on global quality of life [4]. Researchers have suggested that the fatigue might be partly explained by dyspnea due to a mild, lasting pulmonary injury from either chemotherapy, radiotherapy, or both [2, 5]. The association we found between DLCO injury and the reported dyspnea and fatigue on later clinical follow-up supports this explanation.

Older age was found to be significantly related to lesser DLCO recovery. Age was not associated with DLCO injury or recovery in our first report or in any of the others reviewed [5–9, 15, 16]. Therefore, this finding might be due to the more substantial DLCO injury seen in our original study, which made the effect of age on recovery more pronounced, achieving statistical significance. Other studies found that older survivors had poorer HRQOL [2, 26, 27]. As suggested earlier, their lower DLCO recovery may have been a contributory factor.

While aging in general is known to cause a decline in DLCO in healthy adults [28, 29], its impact is seen over decades rather than a few years of follow-up, as in our cohort of mostly middle-aged women. In addition, since all DLCO measurements were performed in the same pulmonary laboratory and compared to the predicted age-specific values, we believe this effect is related more to the treatment than to aging until the last follow-up PFT.

The effects of trastuzumab on DLCO recovery and of DRDI on oncological outcomes did not reach statistical significance, but they are still intriguing and warrant further investigation.

4.1. Strengths and Limitations. We had full information for all patients, and no patient was lost to follow-up. Each patient served as her own control; therefore, the size of our cohort was sufficient for measuring changes over time and interventions. However, the modest size of the cohort limited our ability to perform subgroup analyses and discern effects of various patient and treatment characteristics on pulmonary outcomes. In addition, the follow-up PFTs were performed over a prolonged range of time, making it difficult to quantify the correlation between time until follow-up and pulmonary recovery.

5. Conclusion

DLCO injury after DDC administration is observed in almost all patients with breast cancer. Most patients recover and return to their near-baseline pulmonary function. However, some have a lasting symptomatic DLCO injury, with older patients at higher risk. The significant DLCO injury observed with standard-of-care therapy in patients with potentially curable breast cancer is evident even years afterwards and might play a role in the decrease in overall health and quality of life of survivors. Effects of other breast cancer therapeutics on pulmonary function need to be better elucidated. Practitioners should be aware of these long-term effects of treatment.

Data Availability

The clinical data used to support the findings of this study are included within the article.

Ethical Approval

All treatments given in this study to high-risk early breast cancer patients were according to the standard of care in our center. Additional noninvasive tests performed were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study.

Disclosure

An earlier version of this research was presented as an abstract at the 2018 meeting of the American Society of Clinical Oncology and is published on its website.

Conflicts of Interest

The authors have declared no conflicts of interest.

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Research Article

Relationship between Mammographic Density and Age in the United Arab Emirates Population

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Objective. Higher breast density is a strong, independent risk factor for breast cancer. Breast density varies by age, ethnicity, and geographic area although dense breast tissue has been associated with younger age and premenopausal status. The relationship between breast density and age in women in the United Arab Emirates (UAE) has not been determined. This study evaluated breast density in the UAE population and its relationship with age. *Methods*. Women participating in the national cancer screening program from August 2015 to May 2018 who underwent screening mammography were included. Breast parenchymal density was classified according to the American College of Radiology (ACR) Breast Imaging-Reporting and Data System (BI-RADS) from category a (almost entirely fatty) through d (extremely dense). Subjects were divided into six age groups, and the association between age and breast density was evaluated. *Results*. Of the 4911 women included, 1604 (32.7%), 2149 (43.8%), 1055 (21.5%), and 103 (2.1%) were classified as having categories a–d breast density, respectively. A significant negative correlation was observed between age and breast density category ($p < 0.001$). Women of mean age 44 ± 7 years had the highest breast density, whereas those of mean age 56 ± 14 years had the lowest breast density. Comparisons of Emirati women with Lebanese and Western women showed that breast density was lower in Emirati women than in the other populations. *Conclusions*. To our knowledge, this is the first study to evaluate the relationship between mammographic breast density and age in UAE women. As in other populations, age was inversely related to breast density, but the proportion of Emirati women with dense breasts was lower than in other populations. Because this study lacked demographic, clinical, and histopathological data, further evaluation is required.

1. Introduction

Breast cancer is the most common malignancy among women in developed countries and a major cause of cancer deaths in women worldwide. Among women in countries in the Gulf Cooperation Council (United Arab Emirates (UAE), Bahrain, Saudi Arabia, Oman, Qatar, and Kuwait), the incidence of breast cancer was found to have increased 40% over a 12-year period from 1998 to 2009 [1]. Breast cancer is also the most common form of cancer among Emirati women, accounting for 38.8% of all malignancies in 2014, including both citizens (32.16%) and noncitizens (41.41%) of the UAE [2]. Early detection of breast malignancy has been shown to improve patient prognosis and survival [3].

Screening mammography is an effective tool for detecting early breast cancer, thereby reducing rates of morbidity and mortality [4]. Mammography is currently regarded as the only method appropriate for mass screening. However, higher breast density has been strongly associated with decreased mammographic sensitivity [5]. Breast density is measured by determining the ratio of radiodense epithelium and stroma to radiolucent fatty tissue. Higher breast density on mammography is strongly and reproducibly associated with an increased risk of breast cancer, especially in younger women [6–8]. Although postmenopausal changes in glandular breast tissue reduce breast density in an age-dependent manner.

In addition to being related to breast density, age, as well as race, was reported to be associated with changes in mammographic parenchymal patterns [9]. Breast density can vary by ethnic group and geographic area, for reasons that remain unclear [10, 11]. Thus, measurements of breast density are important for risk assessment and prevention strategies. Moreover, knowledge of the ranges of breast density in particular populations is important when planning screening programs.

The Federal Ministry of Health of the UAE initiated a breast cancer screening program in 1995. More recently, the government of the UAE has promoted early cancer detection programs, with the health authorities of Abu Dhabi and Dubai introducing cancer screening programs in 2008 and 2014, respectively. In 2015, the UAE Ministry of Health launched an official cancer screening initiative for breast, cervical, and colorectal cancers. The programs for breast cancer are in accordance with international guidelines, combining monthly breast self-examination, regular clinical breast examination, and mammography biennially beginning at age 40 years [12–16]. To our knowledge, no study to date has evaluated the distribution of mammographic breast density or the relationship between breast density and age in the UAE population. The present study therefore evaluated breast density and its relationship to age among women in the UAE.

2. Materials and Methods

Women aged ≥40 years participating in the UAE national cancer screening program who underwent screening mammography from August 2015 to May 2018 were included. And, only citizens participated in the UAE national cancer screening program. Mammograms were performed on FDR 3000AWS (Family Health Promotion Center, SKGH, and DHADNA Health Center), FDR 2000AWS (MUHASNAH Health Center, Fujairah Hospital), Selenia Dimensions (RAK Clinic), Senograph DS ADS (Mother & Child Hospital), Lorad Selenia (VEJTHANI Hospital), and Mammo Diagnost DR (LLH) units. Two mammographic images were obtained from each subject, one in the mediolateral oblique and the other in the craniocaudal plane. All screening mammograms were reviewed retrospectively.

Each mammographic report included an assessment of breast density, as defined by the American College of Radiology (ACR) Breast Imaging-Reporting and Data System (BI-RADS). Breast density was divided into four categories, with category a indicating breasts that were predominantly fatty; b indicating scattered fibroglandular density; c indicating heterogeneously dense breasts, which may obscure small masses; and d indicating extremely dense breast tissue, which reduces the sensitivity of mammography. Breast tissues of categories a and b were considered fatty, and those of categories c and d were considered dense. All mammograms were interpreted by a radiologist with over 10 years of experience in breast imaging. Women were also divided into five age groups (40–49, 50–59, 60–69, 70–79, and ≥80 years), and the association between age group and breast density was analyzed, as was the association between breast density and age as a continuous variable. Correlations between age and breast density were assessed by the ANOVA test. The results in Emirati women were compared with those of Lebanese and Western populations, with statistically significant differences determined by Fisher's exact test. All statistical analyses were performed using SPSS software (version 21, SPSS).

3. Results

During the study period, from August 2015 to May 2018, 4911 women underwent mammography. Of these, eight were excluded, three because they were male and five because they lacked BI-RADS density data, with most of the latter having diffuse foreign body granuloma in the breast due to a history of interstitial injection. The remaining 4911 women were considered eligible. Of these 4911 women, 2147 (43.7%), 1727 (35.2%), 803 (16.4%), 198 (4.0%), and 36 (0.7%) were aged 40–49, 50–59, 60–69, 70–79, and ≥80 years, respectively. Mammography results showed that, based on ACR BI-RADS grading, 1604 (32.7%), 2149 (43.8%), 1055 (21.5%), and 103 (2.1%) women were classified into categories a–d, respectively (Table 1).

Analysis of mammographic breast density by age group showed that breast density was normally distributed (Table 2, Figure 1). Spearman's rank-order correlation analysis showed a significant negative correlation between age and breast density category ($p < 0.001$).

The BI-RADS results for the entire screening cohort are summarized in Table 3. Of the 4911 subjects, 4655 (94.8%) had normal or benign screening results, whereas 16 (0.3%) were classified into BI-RADS categories 4 and 5 (Table 3).

These results in Emirati women were compared with published results in Lebanese and Western women [17, 18] (Table 4, Figure 2). These studies also found a negative correlation between breast density and age. However, the percentage of Emirati women with lower breast density was higher than the percentages of Lebanese and Western women over all ages (Figure 3).

4. Discussion

Breast cancer is a major health concern among Emirati women. In the UAE, cancer is the third leading cause of death [19] and breast cancer is the most frequent type of cancer in women [2]. Nevertheless, only 10% of Emirati

Table 1: ACR type results from screening mammography.

ACR BI-RADS density	Number of women	Minimum age in years	Maximum age in years	5% trimmed mean age in years	SD in years
a	1604	40	94	55.9	9.7
b	2149	40	103	52.1	8.3
c	1055	40	78	48.4	6.3
d	103	40	68	45.6	4.8
Total	4911	40	103	52.6	8.9

ACR category a: predominantly fatty (low density); category b: scattered fibroglandular densities (average density); category c: heterogeneously dense (high density); category d: extremely dense (very high density).

Table 2: Distribution of mammographic density with age group.

Age group (years)	ACR category a	ACR category b	ACR category c	ACR category d	Total
40~49	440 (20.5%)	940 (43.8%)	685 (31.9%)	82 (3.8%)	2147
50~59	592 (34.3%)	808 (46.8%)	307 (17.8%)	20 (1.2%)	1727
60~69	420 (52.3%)	329 (41.0%)	53 (6.6%)	1 (0.1%)	803
70~79	127 (64.1%)	61 (30.8%)	10 (5.1%)	0 (0.0%)	198
≥80	25 (69.4%)	11 (30.6%)	0 (0.0%)	0 (0.0%)	36
Total	1604 (32.7%)	2149 (43.8%)	1055 (21.5%)	103 (2.1%)	4911

Figure 1: Distribution of breast density classifications by age groups.

Table 3: BI-RADS results from screening mammography of 4911 patients in the study group.

BI-RADS category	No. (%) of patients	Recommendation
0	240 (4.9)	Recall; additional imaging
1	3448 (70.2)	Routine biennial mammography
2	1185 (24.1)	Routine biennial mammography
3	22 (0.4)	Short-interval follow-up; repeat mammography in 6 months
4	8 (0.2)	Suspicious abnormality; biopsy recommended
5	8 (0.2)	Highly suggestive of malignancy; biopsy recommended

Table 4: Distribution of breast density according to age of UAE, Lebanon, and New York women.

ACR category	Country	Age group (%)			
		40–49	50–59	60–69	≥70
a	UAE	440 (20.5)	592 (34.3)	420 (52.3)	152 (65.0)
	Lebanon	8 (2.3)	30 (10.3)	33 (19.0)	27 (24.8)
	Western (NY)	53 (3.2)	137 (6.3)	200 (12.2)	165 (13.2)
b	UAE	940 (43.8)	808 (46.8)	329 (41.0)	72 (30.8)
	Lebanon	87 (24.8)	128 (43.8)	97 (55.7)	55 (50.5)
	Western (NY)	382 (22.8)	814 (37.1)	710 (43.3)	620 (49.5)
c	UAE	685 (31.9)	307 (17.8)	53 (6.6)	10 (5.1)
	Lebanon	216 (61.5)	124 (42.5)	41 (23.6)	24 (22.0)
	Western (NY)	962 (57.4)	1072 (48.9)	667 (40.7)	407 (32.5)
d	UAE	82 (3.8)	20 (1.2)	1 (0.1)	0 (0.0)
	Lebanon	40 (11.4)	10 (3.4)	3 (1.7)	3 (2.8)
	Western (NY)	278 (16.6)	169 (7.7)	62 (3.8)	60 (4.8)
Total	UAE	2147	1727	803	234
	Lebanon	351	292	174	109
	Western (NY)	1675	2192	1639	1252

Figure 2: Bar graph showing patients' age and categories of breast density by researches.

women undergo breast screening, and over 65% of breast malignancies are diagnosed in advanced stages [20]. It is therefore important to raise awareness and introduce regular screening methods for breast cancer in the UAE.

In many developed countries, breast cancer screening includes mammography performed every 2 years. This screening method has reduced breast cancer mortality rates, especially among women aged 50–69 years, and it is recommended by the European Union and numerous individual countries [21]. Screening mammography is effective in Western women aged >40 years. Its sensitivity and specificity are age-dependent, being higher in older women [22].

Earlier screening has been recommended in Arab populations [23]. Emirati women tend to develop breast cancer at least 10 years earlier than women in Western countries [2, 23, 24]. For example, 25.5% of breast cancers develop in women aged <40 years living in Gulf Cooperation Council countries, compared with <5% in women aged <40 years living in Western countries [1]. The clinicopathological and immunohistochemical features of breast cancer differ in Arab and Western populations [24, 25]. Younger

Figure 3: Comparison in the percentage of females with breast density of 50% and above between our study's results and the studies performed in Lebanon [17] and New York [18].

women tend to have denser breast tissue, with higher ratios of glandular tissue to fat, than older women. This study showed that breast density on mammography was generally lower in Emirati than in Western women over all age groups. The results of screening mammography in the UAE should be adjusted according to local data rather than to international and foreign data. Based on the results of this study, which showed that breast density in Emirati women is lower than in general occidental and Lebanese population, breast density is not by itself or alone responsible for early age breast cancer incidence in the Emirates. Changing in screening mammography programs should take into consideration other factors than breast density in the Emirates to explain early onset incidence of breast cancer. Therefore, nowadays, no change in breast cancer screening program is advisable.

This study had several limitations. First, this study did not include demographic data on the study population, such as history of breast feeding and hormonal replacement therapy, body mass index, menopausal status, parity, and personal and familial history of breast cancer. There were also no clinico-histopathological data. Determining the incidence of breast cancer is required to evaluate the relationship between mammographic breast density and breast cancer risk factors in Emirati women. These sources were difficult to collect the personal data because these were a UAE national cancer screening program. Additional data are required to improve access to breast cancer screening. Therefore, data will be collected and analyzed for follow-up women in hospitals where it is possible to collect data among Emirati women enrolled in the screening program. In addition, the interpretation of mammographic density category may vary among radiologists [26]. The reproducibility of BI-RADS breast density among radiologists may be improved by continued monitoring of their density classifications, with variations among radiologists reduced by the implementation of educational and/or quality assurance measures [26].

Barriers to breast cancer screening have been found to include costs, time concerns, quality of care issues, fear, and medical concerns overlapping with clinical and service quality initiatives. Health system-related barriers have also been identified, including access to insurance, physician recommendation, physician gender, provider characteristics, having a regular provider, fear of the system or procedure, and knowledge of the health system, all of which can be addressed within the UAE [27].

5. Conclusions

To our knowledge, this study is the first to evaluate the relationship between mammographic breast density and age in Emirati women. As in other populations, age was inversely related to breast density, but the proportion of Emirati women with dense breasts was lower than in other populations. The present study indicates that regular screening mammography is effective among women in the UAE.

Although many studies, in various regions of the world, have assessed the epidemiology of breast cancer, few have assessed the incidence of breast cancer among women in the UAE. This paper has significance to provide the breast screening basic data and to determine the relationship between breast density and age in the Emirati women. Our findings indicate the importance of tracking demographic and cancer information in Emirati and Arab women, and the use of that information to establish local screening and diagnostic and therapeutic guidelines.

Data Availability

The demographic and clinical data collected for the purpose of the statistical analysis to support the findings of this study are available from the corresponding author upon request.

Disclosure

This paper was presented as a poster at the 16th St. Gallen International Breast Cancer Conference in Vienna, and the abstract was published in "The Breast; March 2019, supplement 1 page s48."

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Polymorphisms in Survivin (*BIRC5* Gene) Are Associated with Age of Onset in Breast Cancer Patients

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Survivin, encoded by *BIRC5* gene (baculoviral IAP repeat containing 5), belongs to the family of inhibitors of apoptosis proteins (IAPs). In mammalian cells it participates in the control of mitosis, apoptosis regulation, and cellular stress response. Its expression is increased in almost all types of cancers. The aim of this study was to investigate the role of *BIRC5* polymorphisms in breast cancer (BC) and to connect survivin expression with various clinicopathological characteristics of BC patients. Blood and archival tumour tissue samples were collected from 26 BC patients from Croatia. Survivin expression was determined immunohistochemically. *BIRC5* promoter, coding region, and 3'UTR were genotyped. DNA from 74 healthy women was used as control. *BIRC5* polymorphisms and survivin expression were tested against age of onset, histological grade, tumour type and size, lymph node status, oestrogen, progesterone, Her2, and Ki67 status. Numbers of samples with weak, moderate, and strong survivin expression were 9 (33.3%), 11 (40.7%), and 7 (25.9%), respectively. Most patients had nuclear survivin staining (92.6%). High survivin expression was significantly associated with negative oestrogen receptor status (p=0.007) and positive Ki67 expression (p=0.032). Ki67 expression was also positively correlated with histological grade (p=0.0009). Fourteen polymorphisms were found in BC samples, located mostly in promoter and 3'UTR of *BIRC5*. There was no significant difference in the distribution of polymorphisms between BC and control samples. Among clinicopathological characteristics of BC patients, alleles of five *BIRC5* polymorphisms were associated with younger age of onset: c.-644T>C (55.8 years [y] vs. 48.1 y; p=0.006), c.-241C>T (54.2 y vs. 45.0; p=0.029), c.9809T>C (55.8 y vs. 48.1 y; p=0.006), c.-1547C>T (58.3 y vs. 50.9 y; p=0.011), and c.9386T>C (50.8 y vs. 59.5 y; p=0.004). To assess the significance of *BIRC5* polymorphisms and survivin expression as predictive and prognostic biomarkers for BC further research with a larger sample size is needed.

1. Introduction

Breast cancer (BC) is one of the most common malignancies among women worldwide. Despite generally good prognosis for BC patients, there is a wide variation in survival [1]. Some of the common risk factors for BC include age, genetic background, hormonal factors, reproductive and menstrual history, excessive alcohol consumption, radiation, benign breast disease, and obesity [2]. Genetic variation has been shown to affect both susceptibility and prognosis of BC [1]. Genome wide association studies (GWAS) have recently been used to identify new loci for BC, and so far almost

100 loci have been identified [2]. In addition to providing new prognostic markers, genetic determinants of prognosis can also give new biological insight for progression of BC [3]. Survivin, encoded by *BIRC5* (baculoviral IAP repeat containing 5) gene, belongs to the family of inhibitors of apoptosis (IAP) proteins [4]. It is the smallest of eight IAP proteins and contains a single Baculovirus IAP Repeat (BIR), which is the hallmark of these molecules [5, 6]. Survivin is a multifunctional protein and in mammalian cells it participates in at least three cellular processes: the control of mitosis, the regulation of apoptosis, and the cellular stress response [5]. Survivin is strongly expressed during development, while in healthy organisms it is not expressed in differentiated tissues, and its expression is markedly increased in almost all types of cancers (including bladder cancer, lung cancer, breast cancer, stomach, oesophagus, pancreas, liver, uterine, ovarian, and haematological cancers) [7]. Elevated expression of survivin in BC is associated with increased resistance to chemo- and radiotherapy, increased grade, lymph node invasion, and tumour size, as well as decreased survival rate [8]. According to Marsicano et al., breast cancer patients with higher expression of *BIRC5* have significantly greater chance of developing metastasis than patients with lower expression [9]. Survivin is expressed in a cell cycle-regulated manner, being maximally expressed during the G2/M phase, followed by rapid decline of both the mRNA and protein levels at the G1 phase [10]. Several *BIRC5* polymorphisms were shown to be associated with susceptibility (gastric [11], bladder [12], and hepatocellular [13]), survival (colorectal [14] and breast [15]), or age of onset (ovarian cancer [4]). Some of these polymorphisms create binding sites for either transcription factors or regulatory miRNA and potentially can influence survivin levels. Survivin has been proposed as a potential target for anticancer intervention and several survivin inhibitors and survivin-related molecular therapies are in development [16]. The purpose of this preliminary study was to investigate the role of *BIRC5* polymorphisms in BC. The connection between polymorphisms found and protein expression in tumour tissue and their association was also assessed.

2. Materials and Methods

2.1. Study Subjects. This study included BC patients who had their regular check-up at the Eljuga Polyclinic and agreed to participate. Upon signing the consent forms their blood was drawn. 26 patients, diagnosed between 2000 and 2012 and whose archival paraffin embedded tumour tissue was available from archive at the University Hospital for Tumours, University Hospital Centre "Sestre Milosrdnice," were included in the study. One patient had a bilateral BC and both tumours were available for testing. Demographic and clinicopathological data on patients was collected from medical records. Demographic characteristics included age of onset and time since operation, and clinicopathological characteristics included histological grade, tumour size, oestrogen and progesterone receptor status, Her2 status, lymph node status, type, and Ki67 status (determined routinely as part of the diagnostic procedure in the clinic). Two 5-μm sections of tumour tissue were cut from paraffin block for immunohistochemical analysis. DNA samples from 74 healthy women with no history of BC (median age 80, range 65-101) were collected in our previous study [17]. Although usual case-control studies are age matched, in this case it was more appropriate to use elderly healthy population as control. This population is less likely to have cancer predisposing polymorphisms. The study was approved by the Institutional Ethics Committee (EP-6820/13-11).

2.2. Immunohistochemical Staining. Survivin protein was analysed as follows: slides were deparaffinized, prewarmed in Epitope Retrieval Solution (Dako, Glostrup, Denmark) and kept at 95-99°C for 20 min, left to cool for additional 20 min, and then washed in PBS 3x5 min. Endogenous peroxidase was blocked with 3 % H_2O_2/methanol for 10 min and then washed 3x5 min in PBS. The sample was circled by PAP-PEN (Kiyota, Baltimore, MD). Protein block serum-free (DAKO) was added for 10 min.

Immunohistochemical staining was performed using monoclonal rabbit anti-survivin antibody (1:200, 71G4B7, Cell Signalling Technology, Danvers, MA). Following an overnight incubation, the immunodetection was completed using the LSAB Visualization System (DAKO) utilizing 3, 3-diaminobenzidine (DAB) chromogen as substrate, according to the manufacturer's instructions. All sections were counterstained with haematoxylin (DAKO). Negative controls were obtained by omitting the primary antibody.

Stained slides were examined by two experienced pathologists. Samples were scored between 1 and 3 based on survivin expression, taking into account the percentage of positive cells and the staining intensity, as previously described by Xu et al. [18]. Staining intensity was expressed as + (weak), ++ (moderate), and +++ (intense). Percentage of tumor cells positive for survivin staining was categorized as 0 (no positive cells), 1 (<10 %), 2 (10-50 %), and 3 (>50 %). Final score was then generated by multiplying the staining intensity with the percentage of positive cells and ranged from 0 (no stained tumor cells) to 9 (>50 % of intensively stained tumor cells). The final score of 0 was considered as negative (0), 1-3 as weak (1), 4-6 as moderate (2), and 7-9 points as strong (3).

2.3. SNP Selection and Genotyping. Whole coding region of the *BIRC5* gene (including alternative exons 2alpha, 2B, and 3B) was genotyped, including the most common SNPs in *BIRC5* promoter and the 3' untranslated region (3'UTR). Polymorphisms that were selected from the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/snp) are those usually used in association studies (see [4, 10, 13–15, 19–21]). The majority of these studies have found that polymorphisms in 5' and 3' regions have the greatest impact on survivin expression [22] and might contribute to risk of various cancers [21]. Genomic DNA from blood was extracted by the salting out method. Thirteen PCR fragments were analysed using high resolution melting analysis on High-Resolution Melter (HR-1, Idaho Technology, USA) as described by Cvok et al. [17] followed by Sanger sequencing (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, USA). Due to presence of 5 different

Table 1: Patient clinicopathological characteristics.

Characteristic	No. of patients (%)
Age at diagnosis (years)	
< 40	1 (3.8)
40-50	9 (34.6)
50-60	8 (30.8)
> 60	8 (30.8)
Years since operation	
5	8 (30.8)
10	15 (57.7)
15	3 (11.5)
Histological grade	
1	4 (14.8)
2	12 (44.4)
3	10 (37.0)
N.A.*	1 (3.7)
Tumour size	
1 (≤ 2 cm)	20 (74.1)
2 (> 2 ≤ 5 cm)	6 (22.2)
3 (> 5 cm)	1 (3.7)
Oestrogen receptor status	
Positive	21 (77.8)
Negative	6 (22.2)
Progesterone receptor status	
Positive	16 (59.3)
Negative	11 (40.7
Her2 status	
Positive	2 (7.4)
Negative	22 (81.5)
N.A.	3 (11.1)
Lymph node status	
Positive	11 (40.7)
Negative	16 (59.3)
Pathologic Characteristics	
Invasive ductal carcinoma	22 (81.5)
Medullary carcinoma	2 (7.4)
Invasive lobular carcinoma	1 (3.7)
Mixed invasive ductal/lobular carcinoma	1 (3.7)
Papillary carcinoma	1 (3.7)
Ki67 status	
Positive	12 (44.4)
Negative	12 (44.4)
N.A.	3 (11.1)

∗ Patient data not available.

polymorphisms in the PCR product of exon 4, it was directly sequenced. PCR primer sequences and cycling conditions are listed in Supplementary Table 1.

2.4. Statistical Analysis. Genotype and allele frequencies of patients and controls were compared using $\chi 2$ test. Deviation from the Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were calculated using the online tool SHEsis [http://analysis.bio-x.cn/myAnalysis.php] [23]. The $\chi 2$ test was used for testing the association between genetic data and categorical clinicopathological variables. Independent samples t-test and one-way analysis of variance with Tukey-Kramer post hoc test were used for testing the difference between continuous (age of onset and logarithmic transformed tumour size) and categorical variables. Two-tailed p-values less than 0.05 were considered statistically significant. Statistical analysis was performed using MedCalc v18.2.1 (MedCalc Software bvba).

Table 2: Levels and localization of survivin expression.

Characteristic	No. of samples (%)
Survivin immunoreactivity score	
weak	9 (33.3)
moderate	11 (40.7)
strong	7 (25.9)
Survivin cellular localization	
cytoplasmic	1 (3.7)
nuclear	25 (92.6)
cytoplasmic+nuclear	1 (3.7)

3. Results and Discussion

3.1. Patient Characteristics. A total of 26 patients with BC were recruited in this study. The main clinical and histopathological features are summarized in Table 1. The median age of patients at diagnosis was 53 years (range 37-69): only one patient (3.8%) was younger than 40 years, 34.6% were between 40 and 50 years, 30.8% were between 50 and 60, and 30.8% were older than 60. The majority of patients (96.2%) were still alive at the end of the study so survival analysis was not performed.

Tumour size was associated with positive lymph nodes (p=0.004). Histological grade was associated with Ki67 expression (p=0.0009). All samples with histological grade 1 were negative for Ki67, while only one sample with histological grade 3 was negative for Ki67. Progesterone receptor (PR) and oestrogen receptor (ER) status were weakly positively associated (0.053). These results are in agreement with previously published international data [24–27].

3.2. Survivin Expression. Survivin expression was analysed in 27 BC samples from 26 patients using immunohistochemical staining. Samples were scored between 1 and 3 based on survivin expression, taking into account the percentage of positive cells and the staining intensity (Figure 1). The numbers of samples exhibiting survivin expression with a score of 1 (weak), 2 (moderate), and 3 (strong) were 9 (33.34%), 11 (40.72%), and 7 (25.94%), respectively (Table 2). The majority of patients had nuclear survivin staining (92.6%), so we combined all the samples together for the statistical evaluation.

The high nuclear staining of survivin is different than most published data, where the nuclear staining is mostly lower than cytoplasmic [18, 28–31] and only rarely has been described as equal or higher than cytoplasmic [32, 33].

Figure 1: Examples of scored staining intensities: (a) negative control, (b) score 1, (c) score 2, and (d) score 3.

3.3. Association of Survivin Expression with Clinicopathological Parameters. Clinicopathological findings (age at diagnosis, time since operation, histological grade, tumour size, oestrogen and progesterone receptors, Her2 status, lymph node status, and Ki67 status) were compared with the immunohistochemical characterization of survivin expression. Since the majority of samples (22 of 27 samples, 84.6%) were ductal BC all samples were grouped together. The statistical analyses revealed a significant association between survivin expression and ER status. High survivin expression was significantly associated with negative ER status (p=0.007). No samples with low survivin expression (score 1), 18.2% samples with medium expression (score 2), and 57.1% of samples with high survivin expression (score 3) had negative ER status (Figure 2). Survivin expression was weakly associated with negative PR status (p=0.051). Furthermore, another statistically significant association was confirmed between survivin expression and Ki67 expression. High survivin expression was associated with positive Ki67 expression (p=0.032).

There is controversial data reported about association between high survivin expression and negative ER and/or PR status in BC. While some studies [34, 35] found the same association as here, other studies report these parameters as not significant [32, 36, 37]. The similar situation is with the association between high survivin expression and Ki67 expression. While some studies [38] showed the same results as here, others showed there was no significant association between survivin and Ki67 [31, 39].

Figure 2: Association between survivin expression and ER status. Survivin expression was ranked weak, moderate, and strong, and percentage of ER negative and ER positive samples per group is shown.

3.4. BIRC5 Polymorphisms in Breast Cancer Patients. Fifteen different polymorphisms were found in the constitutional DNA of 26 BC patients and 74 healthy controls.

The whole *BIRC5* gene coding region and selected promoter and 3'UTR regions were investigated for selected ten polymorphisms. One of the ten polymorphisms (c.-235G>A) was not found in BC patients. Polymorphisms and their frequencies are listed in Table 3.

According to the TRANSFAC database (http://genexplain.com/transfac), promoter and 5'UTR region of *BIRC5* gene

Table 3: Comparison of minor allele frequencies of *BIRC5* polymorphisms between breast cancer patients and healthy control samples. Nucleotide changes are named according to the literature.

gene region	SNP ID number	nucleotide change	minor allele frequency controls (n/N, %)∗	minor allele frequency BC samples (n/N, %)∗	p (for allele frequencies)
promoter	rs3764383	c.-1547C>T∗∗	37/148 (25.0)	16/52 (30.8)	0.466
promoter	rs8073903	c.-644T>C	49/148 (33.1)	18/52 (34.6)	0.865
promoter	rs8073069	c.-625G>C	33/148 (22.3)	13/52 (25.0)	0.704
promoter	rs17878731	c.-267G>A	1/148 (0.7)	1/52 (1.9)	0.453
promoter	rs17878467	c.-241C>T	16/148 (10.8)	6/52 (11.5)	1.000
promoter	rs17887126	c.-235G>A	2/148 (1.3)	0/52 (0)	
5'UTR	rs9904341	c.-31G>C	55/148 (37.2)	18/52 (34.6)	0.867
intron 2	rs4789551	c.221+209T>C	7/148 (4.7)	1/52 (1.9)	0.683
exon 4	rs2071214	c.9194G>A∗∗∗	5/148 (3.4)	1/52 (1.9)	1.000
3'UTR	rs17885521	c.9288G>C	3/148 (2.0)	1/52 (1.9)	1.000
3'UTR	rs17882627	c.9342G>A	2/148 (1.3)	2/52 (3.8)	0.278
3'UTR	rs2239680	c.9386T>C	34/148 (23.0)	14/52 (26.9)	0.575
3'UTR	rs17882139	c.9387_9388insAA	3/148 (2.0)	2/52 (3.8)	0.606
3'UTR	rs1042489	c.9809T>C	53/148 (35.8)	18/52 (34.6)	1.000
3'UTR	rs2661694	c.10611C>A	38/148 (25.7)	11/52 (21.1)	0.578

∗ n – number of minor alleles, N – number of analyzed alleles
∗∗C is the minor allele
∗∗∗G is the minor allele.

Figure 3: Structure of *BIRC5* gene with positions of analyzed polymorphisms. Positions of known binding sites for transcription factors and miRNA are shown according to TRANSFAC database [40]. Chromosome 17 positions are numbered according to human genome build GRCh38/hg38.

contain 29 binding sites for 14 different transcription factors. Chromosome positions of the detected polymorphisms revealed that polymorphisms rs17878467 (c.-241C>T) and rs17887126 (c.-235G>A) are located in the shared binding site of transcription factors EGR1 and SP1 (Figure 3). 3'UTR region also contains 16 binding sites for 11 different miRNAs, and only polymorphism rs1042489 (c.9809T>C) is located in the binding site of hsa-miR-708-5p (Figure 3) [40].

Fourteen different polymorphisms were found in BC samples while in controls one more polymorphism was

Figure 4: Pairwise linkage disequilibrium (LD) of eight *BIRC5* sequence variants with highest minor allele frequency. The location of each sequence variant along the *BIRC5* gene is relative to the real nucleotide position. The number in each diamond indicates the intensity of LD (r^2) between respective pairs of sequence variants. The LD strength is also represented by shades of grey (0 [white] < r^2 < 1 [black]). (a) Controls. (b) Cases.

present (c.-235G>A). The majority of detected polymorphisms were located either upstream or downstream of the coding region of the gene, and only one in the coding region. In addition to ten polymorphisms selected for this study, five more rare polymorphisms were found: one in the promoter (c.-267G>A), one in intron 2 (c.221+209T>C), and 3 in the 3'UTR (c. 9288G>C, c.9342G>A, and c.9387_9388insAA). There was no significant difference in genotype or allele frequencies between BC and control samples.

The observed allele frequencies for most of the polymorphisms are in accordance with previously observed frequencies for either global or European populations [15, 19, 20, 22, 41–43], or in NCBI database (http://www.ncbi.nlm.nih.gov), and only for c.221+209T>C the frequency was lower in this study than previously observed [41, 44]. c.-235G>A polymorphism found only in healthy controls is known to create a new "alternative" binding site for the transcription factor GATA-1 and is suggested to increase the BC aggressiveness [45]. Since most of the patients in this study are still alive, it is possible that this is a reason this polymorphism was not found in the patient group.

Analysis showed there is a difference in number of polymorphisms in linkage disequilibrium (LD) between BC samples and controls. BC cases showed increased nonrandom association of alleles at multiple loci. In case of c.-644C>T and c.9809T>C, these two polymorphisms always appear in the same combination in all tested BC samples showing complete LD (r^2=0.99) (Figure 4). Only one pair of polymorphisms, c.9386T>C and c.10611C>A, shows decreased association in BC samples compared to control samples.

LD between various *BIRC5* polymorphisms have often been reported in different populations: some observed here, but also others. LD between c.-1547C>T and c.9368 found here has been reported by Shi et al. [15], as were c.-1547C>T with c.10611C>A and c.9386T>C with c.10611C>A. The latter one was also reported by Pu et al. [46]. LD between c.-644T>C and c.-625G>C was reported twice, by Jang et al. [19] and Yang et al. [42], and between c.-625G>C and c.9809T>C once [47]. Interestingly, the polymorphisms in total LD, c.-644C>T and c.9809T>C, found in BC patients have not been described before.

3.5. Association of BIRC5 Polymorphisms with Survivin Expression and Clinicopathological Parameters. *BIRC5* polymorphisms were not associated with survivin expression, but several polymorphisms were found to be associated with age of onset. Genotype frequencies of polymorphisms c.-644T>C, c.9386T>C, and c.9809T>C were significantly associated with age of onset, while c.-1547C>T was weakly associated. Allele frequencies of these four, as well as SNP c.-241C>T, were also associated with age of onset (Table 4). When genotype frequencies were plotted against age of onset, a clear trend could be seen for the five polymorphisms (Figure 5) (the results are presented as in Han et al.'s study [4]).

For the c.-1547C>T polymorphism, the minor allele T was associated with earlier age of onset. The same trend is visible for the c.-644C>T allele and the c.9089C>T allele, where in both cases the minor C allele is associated with earlier age of onset. For the c.9386T>C allele, the major allele T is associated with earlier age of onset. For the final, c.-241C>T polymorphism, where the difference was significant only on allele but not on genotype frequencies, the minor allele T also seems to be associated with earlier age of onset but the

Figure 5: Association of age of onset with the significantly associated polymorphisms. (a) c.-1547C>T. (b) c.-644T>C. (c) c.-241C>T. (d) c.9386T>C. (e) c.9809T>C.

number of samples in the TT group is too low to make this comparison.

Until now, only two studies showed association between *BIRC5* polymorphisms and age of onset. Han et al. [4] found significant connection of age of onset for the c.-1547A>G and less significantly c.-31G>C polymorphisms in ovarian cancer, while Hmeljak et al. [20] found that c.-241C>T was associated with the age of diagnosis of malignant pleural mesothelioma patients, but not with total survival. These results are comparable with our results in BC, where we show that the minor allele of the c.1547A>G polymorphism is also associated with age of onset, and in our case the results for the c.-241C>T are significant when comparing allele, but not for genotype frequencies. This discrepancy may be due to a small number of samples with the TT genotype (only one in our dataset). In BC, amplifications in *BIRC5* gene were also associated with the younger age of onset [48].

Table 4: Association of genotype and allele frequencies of polymorphisms with age of onset.

polymorphism			mean±SD (years)	p-value
c.-1547C>T∗	genotype	CC	62.3±6.5	0.054
		CT	55.8±8.1	
		TT	49.0±10.1	
	allele	C	58.3±7.9	**0.011**
		T	50.9±9.8	
c.-644T>C	genotype	TT	59.4±8.2	**0.006**
		TC	50.7±8.6	
		CC	39.0±2.8	
	allele	T	55.8±9.2	**0.006**
		C	48.1±9.1	
c.-241C>T	genotype	CC	54.7±10.0	0.143
		CT	49.0±4.5	
		TT	37.0±0.0	
	allele	C	54.2±9.6	**0.029**
		T	45.0±7.1	
c.9386T>C	genotype	TT	49.1±9.3	**0.029**
		TC	57.4±8.4	
		CC	62.3±6.5	
	allele	T	50.8±9.6	**0.004**
		C	59.5±7.6	
c.9809T>C	genotype	TT	59.4±8.2	**0.006**
		TC	50.7±8.6	
		CC	39.0±2.8	
	allele	T	55.8±9.2	**0.006**
		C	48.1±9.1	

∗ C is the minor allele.

4. Conclusions

This was the first study investigating the possible role of *BIRC5* polymorphisms in breast cancer etiology conducted in Croatia. In our study no association of any of *BIRC5* polymorphism with BC or level of survivin expression was observed. High survivin expression was significantly associated with negative ER status. However, as many as five *BIRC5* polymorphisms were associated with the age of onset, a phenomenon which was previously observed only in ovarian cancer patients. Two of those five polymorphisms (c.-644T>C and c.9809T>C) were also shown to be in complete LD in breast cancer patients. We are aware that the sample size of this study was limited, but it should be noted that the allele frequencies for polymorphisms found were in accordance with previous reports, except for c.-235G>A, which was not present in BC samples. To assess the significance of *BIRC5* polymorphisms and survivin expression as predictive and prognostic biomarkers for BC further research with a larger sample size is needed.

Data Availability

This is not applicable, since there are no big datasets connected with this article.

Disclosure

Results published in this paper were presented as poster at HDIR-5: The Fifth Meeting of the Croatian Association for Cancer Research with International Participation, Zagreb in November, 2018.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Ilona Sušac and Petar Ozretić are equally contributing authors.

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Supplementary Materials

Supplementary Table 1: PCR primer sequences and cycling conditions. *(Supplementary Materials)*

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Research Article

Factors Associated with Initial Mode of Breast Cancer Detection among Black Women in the Women's Circle of Health Study

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Mammogram-detected breast cancers have a better prognosis than those identified through clinical breast exam (CBE) or through self-detection, primarily because tumors detected by mammography are more likely to be smaller and do not involve regional nodes. In a sample of 1,322 Black women, aged 40-75 years, diagnosed with breast cancer between 2002 and 2016, we evaluated factors associated with CBE and self-detection versus screening mammogram as the initial mode of breast cancer detection, using multivariable logistic regression models. Compared with screening mammogram, history of routine screening mammogram (OR 0.20, 95% CI: 0.07, 0.54) and performance of breast self-examination (BSE) (OR 0.31, 95% CI: 0.13, 0.74) before diagnosis were associated with lower odds of CBE as the initial mode of detection, while performance of CBEs before diagnosis (OR 11.04, 95% CI: 2.24, 54.55) was positively associated. Lower body mass index (<25.0 kg/m^2 vs. ≥35.0 kg/m^2: OR 2.46, 95% CI: 1.52, 3.98), performance of BSEs before diagnosis (less than once per month: OR 4.08, 95% CI: 2.45, 6.78; at least monthly: OR 4.99, 95% CI: 3.13, 7.97), and larger tumor size (1.0-2.0 cm vs. <1.0 cm: OR 2.92, 95% CI: 1.84, 4.64; >2.0 cm vs. <1.0 cm: OR 6.41, 95% CI: 3.30, 12.46) were associated with increased odds of self-detection relative to screening mammogram. The odds of CBE and self-detection as initial modes of breast cancer detection among Black women are independently associated with breast care and breast cancer screening services before diagnosis and with larger tumors at diagnosis.

1. Introduction

Although breast cancer incidence rates have been historically lower among African American/Black (hereafter "Black") women than White women [1–5], recent data showed that incidence rates among these groups converged as of 2012 [6]. However, rates of incidence at ages <45 years [7] and mortality among Black women continue to be higher than among Whites [3–10]. National data from 2010-2014 [11, 12] demonstrated that breast cancer mortality was at least 41% higher among Blacks than Whites.

There are currently conflicting breast cancer screening guidelines, published by the United States Preventative Services Task Force (USPSTF) [13] and the American Cancer Society (ACS) [14], for women at average risk. The USPSTF recommends biennial screening among women aged 50-74 who are at average risk of breast cancer and the decision to begin screening prior to age 50 should be an individual one [13]. In contrast, the ACS strongly recommends annual mammography screening for women aged 45-54 and biennial mammography screening starting at age 55 [14]. Similar to the USPSTF position on mammography screening beginning

at age 40, the ACS also recommends that women should have the choice to begin annual screening at this age. In 2009, the USPSTF concluded that there was insufficient evidence regarding the potential benefits and harms of clinical breast exams (CBEs) among women age 40 and older and recommended against teaching breast self-examination (BSE). The most recent USPSTF breast cancer screening guidelines did not update this language [13]. The ACS does not recommend CBE for breast cancer screening among average-risk women of any age and also recommends against systematic instruction of BSE, but suggests that all women should know how their breasts normally look and feel so that they are able to report noticeable changes to their healthcare provider in a timely manner [14].

Women's knowledge and awareness of the importance of mammography screening, as well as of breast cancer signs and symptoms are crucial, especially given changing and sometimes conflicting screening recommendations and evidence showing that initial mode of detection may be an independent prognostic factor for breast cancer. Breast cancers that are initially self-detected are commonly found to share more aggressive phenotypic characteristics (e.g., higher grade, hormone receptor negative, larger tumor size, positive lymph node status, and triple-negative breast cancer [TNBC] subtype) compared to those initially clinically detected through screening mammography or during routine physical exams performed by a healthcare provider [15–24]. Mammogram-detected breast cancers generally have more favorable clinicopathologic characteristics (e.g., lower grade, hormone receptor positive status, smaller tumor size, negative lymph node status, and luminal A subtype) than those originally detected through other means (e.g., self-detection, detection by partner, etc.) [15–25]. Evidence indicates that particular sociodemographic factors also differ when comparing women with screen-detected vs. self-detected breast cancers, including age, race/ethnicity, and educational attainment [23, 26]. Furthermore, evidence suggests that patients with mammogram-detected breast cancers have improved survival rates compared to those with self-detected cancers, specifically among those diagnosed with luminal A breast cancers [15, 16, 19, 22].

As there is currently a dearth of research regarding the factors associated with initial mode of breast cancer detection across diverse populations, the aim of this study was to identify specific factors (with a focus on sociodemographic characteristics, breast care characteristics prior to breast cancer diagnosis, and breast tumor clinicopathologic features at diagnosis) that might be associated with self-reported mode of initial breast cancer detection among a cohort of Black women with breast cancer.

2. Materials and Methods

2.1. Study Sample. We conducted case-case analysis of incident, primary breast cancers diagnosed among Black women 40-75 years, who were enrolled in the Women's Circle of Health Study (WCHS). As previously described [23, 27–29], WCHS is a case-control study conducted in metropolitan New York City (NYC) and ten counties in New Jersey (NJ). Breast cancer cases with histologically confirmed ductal carcinoma in situ or invasive breast cancer, who self-identified as either Black/African American or White/European American, were aged 20-75 years, were able to complete an interview in English, and had no history of cancer except nonmelanoma skin cancer, were eligible to participate. Recruitment in NYC was conducted between January 2002 and December 2008. Case identification was performed through hospitals within the 5 boroughs with the largest referrals for Black women. Recruitment in NJ began in March 2006 and is ongoing. Breast cancer cases residing in ten NJ counties are identified through rapid case ascertainment by the NJ State Cancer Registry (NJSCR). As of the start of the present analysis, approximately 83% of the breast cancer cases identified by NJSCR were deemed eligible for study participation and of these approximately 51% agreed to be contacted by research staff. Of those contacted, approximately 83% consented to participate in the study and completed the study interview. In this analysis, we included 1,322 Black breast cancer cases diagnosed between 2002 and 2016, who were 40-75 years old, were recruited in NYC and NJ, and had completed the baseline interview, including questions ascertaining their initial mode of breast cancer detection. This study was approved by the Institutional Review Boards of all participating institutions and all study participants provided written informed consent prior to enrollment in the study.

2.2. Data Collection. Data collection for the WCHS was conducted through in-person, interviewer-administered questionnaires at approximately 6-9 months after breast cancer diagnosis, assessing data relevant to the time of diagnosis as well as the time period of approximately 12 months before breast cancer diagnosis. Relevant to the current analysis, the baseline study questionnaire ascertained information on sociodemographic characteristics as well as established and probable breast cancer risk factors, including: family and personal medical history, reproductive history and hormone therapy (HT) use, cancer screening characteristics, and lifestyle exposures (e.g., tobacco smoke exposure, alcohol consumption, and physical activity). Anthropometric measurements (height, weight, and waist and hip circumference measures) were also taken at the in-person, baseline interview using standardized protocols and instruments [29]. Breast tumor clinicopathologic data were collected through medical and pathology records obtained from hospitals where breast cancer care was received.

Breast care characteristics approximately 12 months before breast cancer diagnosis were ascertained in the baseline questionnaire with the following questions: (1) Has a doctor ever recommended that you have a routine mammogram before your breast cancer diagnosis (yes, no)? (2) Before your diagnosis, had you ever had a routine mammogram (yes, no)? (3) Before your diagnosis, at what age or what year did you have your last routine mammogram? (4) Before your diagnosis, did you examine your breasts for lumps (yes, no)? (5) Before your diagnosis, did your healthcare provider examine your breasts for lumps (yes, no)? (6) How was your

breast cancer first found (routine self-exam, accidental self-discovery, accidental discovery by a partner, routine physical exam by a doctor, routine screening mammogram, other)? Participants who reported "other" were excluded from subsequent analysis (n=321). Interval between breast cancer diagnosis and most recent routine screening mammogram was calculated as the difference (in years) between diagnosis age and age at last routine mammogram or as the difference (in years) between diagnosis date and date at last routine mammogram. Initial mode of breast cancer detection was classified into three categories: (1) screening mammogram (detected initially by a routine screening mammogram); (2) CBE (detected initially through CBE performed by a healthcare provider or through routine physical exam performed by a healthcare provider); and (3) self-detected (initially detected/palpated through routine BSE, through accidental self-discovery, or through accidental discovery by partner).

2.3. Data Analysis. Descriptive statistics were used to describe the study sample, overall and by initial mode of breast cancer detection. Chi-square tests (for categorical variables) and ANOVA models (for continuous variables) were used to compare sociodemographic, reproductive characteristics, medical history, breast care, and tumor characteristics, overall and by mode of breast cancer detection. Separate logistic regression models for each variable, adjusting for age, were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) of the overall associations between covariates of interest and initial mode of breast cancer detection. All variables that were significant in the age-adjusted analyses were included in a backward elimination procedure to obtain a final multivariable model, which was also age-adjusted. Any variable with $P > 0.10$ was eliminated during the model selection procedure. The primary analysis focused on the associations of interest among women age 40-75 years. Sensitivity analysis was performed to assess the same associations among the subset of women age 50-75 years. Additionally, age-stratified multivariable analysis was performed separately for women who were 40-49, 50-59, and 60-75 years old. All reported P values were two-sided, and $P \leq 0.05$ was considered statistically significant. All analyses were performed using SAS v9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Sociodemographic Characteristics of Study Sample and Differences by Initial Mode of Breast Cancer Detection. Sociodemographic characteristics, overall and by initial mode of breast cancer detection, are shown in Table 1. Among the 1,643 Black WCHS participants enrolled, 1,322 women age 40-75 years, with initial mode of detection classified as screening mammogram, CBE, or self-detection were included in the final analytic sample. Among this sample, 725 (54.8%) reported that their breast cancer was initially detected through screening mammogram, 63 (4.8%) through CBE, and 534 (40.4%) through self-detection. In the overall sample, approximately one-quarter of study participants was diagnosed with breast cancer at age <50 years. Approximately 44% was married or living as married, 35% had a college education or above, and 36% reported an annual household income of ≥$70,000. Nearly 60% percent reported having private health insurance, approximately 10% and 15% reported being covered by Medicaid or Medicare, respectively, 10% was uninsured, and about 6% had some "other" type of health insurance (e.g., school-provided, national government-provided [foreign country], health center care, health savings account, and Veterans Affairs coverage).

Only age ($P < 0.001$) and type of primary health insurance at diagnosis ($P < 0.001$) significantly differed by initial mode of breast cancer detection. The mean age at diagnosis among women whose breast cancers were detected through screening mammogram was approximately 57 years, while that of women reporting CBE and self-detection were 51 years and 50 years, respectively. In terms of insurance status at diagnosis, while there were similarly large proportions of women with private insurance across all modes of detection, the proportion of women who reported being uninsured was larger among those with self-detected breast cancers (14%) relative to those with mammogram-detected (7%) and CBE-detected (8%) cancers.

3.2. Reproductive Characteristics and Medical History of the Study Sample and Differences by Initial Mode of Breast Cancer Detection. As shown in Table 2, 61% of the overall study sample was postmenopausal, almost half (47%) reported being 12-13 years old at menarche, most (83%) had children, among whom 63% was <25 years old at their first live birth and approximately 46% reported having a history of breastfeeding. Almost 30% of the sample had a history of oral contraceptive use and a small proportion reported a history of postmenopausal HT use (17.5%). Less than 20% reported having a family history of breast cancer, and about half of all participants was obese. The distribution of menopausal status, HT use, and BMI differed significantly by mode of detection (all P-values <0.001). The proportion of postmenopausal women was smaller among those who reported their mode of detection was CBE or self-detection, relative to those who reported mammography-detection (43% and 48%, respectively, vs. 72%). Similarly, the proportion of women with a history of HT use was smaller among the CBE-detected and self-detected groups, relative to mammography-detected (10% and 11%, respectively, vs. 23%). Conversely, the proportions of women with BMI <25.0 kg/m^{2}in the CBE-detected and self-detected groups were larger than among those in the mammography-detected group (25% and 26%, respectively, vs. 15%).

3.3. Breast Care Characteristics Prior to Breast Cancer Diagnosis and Tumor Clinicopathologic Features of the Study Sample and Differences by Initial Mode of Breast Cancer Detection. As shown in Table 3, the distributions of all breast care characteristics and breast tumor clinicopathologic features significantly differed by mode of detection. In brief, women who reported CBE or self-detection as the initial mode of breast cancer detection had more unfavorable breast care

Table 1: Sociodemographic characteristics of Black women age 40-75 years in the Women's Circle of Health Study (WCHS), overall and by initial mode of breast cancer detection.

	Overall (N = 1322) *N (%)*	Screening mammogram (n = 725) *n (%)*	Clinical breast exam (n = 63) *n (%)*	Self-detection (n = 534) *n (%)*	*P*-value
Age at diagnosis (years), mean±SD	54.19±10.7	57.35±9.2	51.44±11.1	50.17±11.1	*<0.001*
Age at diagnosis (years)					*<0.001*
40-49	325 (24.6)	147 (20.3)	17 (27.0)	161 (30.2)	
50-59	431 (32.6)	248 (34.2)	17 (27.0)	166 (31.1)	
60-75	449 (34.0)	318 (43.9)	17 (27.0)	114 (21.4)	
Marital status[a]					0.494
Married or living as married	576 (43.6)	326 (45.0)	25 (39.7)	225 (42.1)	
Unmarried	746 (56.4)	399 (55.0)	38 (60.3)	309 (57.9)	
Education					0.390
High school/vocational/technical graduate or below	538 (40.7)	311 (42.9)	25 (39.7)	202 (37.8)	
Some post-secondary education	327 (24.7)	167 (23.0)	13 (20.6)	147 (27.5)	
College graduate or above	456 (34.5)	246 (33.9)	25 (39.7)	185 (34.6)	
Annual household income[b]					0.224
<$70,000	765 (57.9)	400 (55.2)	37 (58.7)	328 (61.4)	
≥$70,000	480 (36.3)	282 (38.9)	21 (33.3)	177 (33.2)	
Primary health insurance at diagnosis[c]					*<0.001*
Private	775 (58.6)	413 (57.0)	39 (61.9)	323 (60.5)	
Medicaid	130 (9.8)	65 (9.0)	8 (12.7)	57 (10.7)	
Medicare	193 (14.6)	140 (19.3)	10 (15.9)	43 (8.1)	
Uninsured	131 (9.9)	51 (7.0)	5 (7.9)	75 (14.0)	
Other	81 (6.1)	50 (6.9)	1 (1.6)	30 (5.6)	
Missing	12 (0.9)	6 (0.8)	0 (0.0)	6 (1.1)	

[a]The unmarried category is composed of those who reported being single/never married, separated, divorced, or widowed.

[b]$70,000 is the median income among households in New Jersey and was used as the cut-point to dichotomize the income variable.

[c]The "other" insurance category includes forms of insurance reported by WCHS participants that did not fall into one of the four main categories above and included the following types of insurance coverage: school-provided, national government-provided [foreign country], spousal insurance, health center care, charity care, health savings account, and Veterans Affairs coverage.

Table 2: Reproductive characteristics and medical history of Black women aged 40-75 years in the WCHS, overall and by initial mode of breast cancer detection.

	Overall (N = 1322) *N (%)*	Screening mammogram (n = 725) *n(%)*	Clinical breast exam (n = 63) *n(%)*	Self-detection (n = 534) *n(%)*	*P*-value
Menopausal status					*<0.0001*
Premenopausal	516 (39.0)	203 (28.0)	36 (57.1)	277 (51.9)	
Postmenopausal	806 (61.0)	522 (72.0)	27 (42.9)	257 (48.1)	
Age at menarche (years)					0.324
<12	381 (28.8)	212 (29.2)	11 (17.5)	158 (29.6)	
12-13	618 (46.8)	338 (46.6)	32 (50.8)	248 (46.4)	
>13	323 (24.4)	175 (24.1)	20 (31.8)	128 (24.0)	
Parity					0.428
Nulliparous	231 (17.5)	117 (16.1)	9 (14.3)	105 (19.7)	
1-2 children	679 (51.4)	377 (52.0)	31 (49.2)	271 (50.8)	
≥3 children	412 (31.2)	231 (31.9)	23 (36.5)	158 (29.6)	
Age at first birth (years)[a]					0.605
<25	687 (63.0)	382 (62.8)	30 (55.6)	275 (64.1)	
25-30	242 (22.2)	130 (21.4)	15 (27.8)	97 (22.6)	
>30	162 (14.9)	96 (15.8)	9 (16.7)	57 (13.3)	
History of breastfeeding[a]					0.458
No	592 (54.5)	336 (55.5)	32 (59.3)	224 (52.3)	
Yes	495 (45.5)	269 (44.5)	22 (40.7)	204 (47.7)	
History of oral contraceptive use					0.685
No	933 (70.6)	505 (69.7)	42 (66.7)	386 (72.3)	
Yes	388 (29.4)	219 (30.2)	21 (33.3)	148 (27.7)	
History of hormone therapy use					*<0.0001*
No	1084 (82.0)	556 (76.7)	57 (90.5)	471 (88.2)	
Yes	231 (17.5)	166 (22.9)	6 (9.5)	59 (11.1)	
Family history of breast cancer					0.733
No	1075 (81.3)	584 (80.6)	52 (82.5)	439 (82.2)	
Yes	247 (18.7)	141 (19.5)	11 (17.5)	95 (17.8)	
Body mass index (kg/m^2)					*<0.001*
<25.0	261 (19.7)	105 (14.5)	16 (25.4)	140 (26.2)	
25.0-29.99	389 (29.4)	226 (31.2)	17 (27.0)	146 (27.3)	
30.0-34.99	312 (23.6)	189 (26.1)	14 (22.2)	109 (20.4)	
≥35.0	359 (27.2)	204 (28.1)	16 (25.4)	139 (26.0)	

[a]Age at first birth and history of breastfeeding data are among parous women only.

characteristics before diagnosis relative to those who reported that their breast cancer was detected through screening mammography. This included lower rates of receiving a doctor's recommendation for mammography, lower rates of ever having a mammogram, and longer time intervals between breast cancer diagnosis and most recent routine screening mammogram (P<0.0001). It should be noted that 18% of the study sample were outside the recommended screening interval, having an interval between diagnosis and the most recent routine screening mammogram that was longer than 2 years. Among women with CBE-detected and self-detected breast cancers, the proportions of higher grade, regional/distant SEER stage, larger size, positive lymph node status, presence of lymphovascular invasion, ER-negative status, and triple-negative subtype were larger relative to those with mammography-detected breast cancers, with women with self-detected cancers having the highest proportions (all *P*-values <0.001).

3.4. Age-Adjusted Associations between Sociodemographic, Reproductive, Clinical, Breast Care, and Tumor Clinicopathologic Characteristics with Initial Mode of Breast Cancer Detection. In the age-adjusted models (Table 4), premenopausal status (vs. postmenopausal status) was associated with increased odds of CBE-detection and younger age at menarche (<12 vs. >13 years) was associated with decreased odds of CBE-detection relative to detection by mammogram. Lower income (<$70,000 vs. ≥$70,000), being uninsured (vs. privately insured), and having a BMI <25.0 kg/m^2 (vs. BMI

Table 3: Breast care characteristics prior to breast cancer diagnosis and tumor clinicopathologic features among Black women aged 40-75 years in the WCHS, overall and by initial mode of breast cancer detection.

	Overall (N = 1643) *N (%)*	Screening mammogram (n = 725) *n(%)*	Clinical breast exam (n = 63) *n(%)*	Self-detection (n = 534) *n(%)*	*P*-value
Breast care characteristics prior to breast cancer diagnosis					
Ever received a doctor's recommendation for a mammogram before breast cancer diagnosis					*<0.001*
No	274 (20.7)	103 (14.2)	16 (25.4)	155 (29.0)	
Yes	1045 (79.1)	620 (85.5)	47 (74.6)	378 (70.8)	
Ever had a routine screening mammogram before breast cancer diagnosis					*<0.001*
No	174 (13.2)	37 (5.1)	16 (25.4)	121 (22.7)	
Yes	1148 (86.8)	688 (94.9)	47 (74.6)	413 (77.3)	
Interval between breast cancer diagnosis and most recent routine screening mammogram					*<0.001*
<1 year	112 (8.5)	66 (9.1)	4 (6.4)	42 (7.9)	
1 year	565 (42.7)	382 (52.7)	17 (27.0)	166 (31.1)	
>1 year	377 (28.5)	186 (25.7)	17 (27.0)	174 (32.6)	
Unknown	268 (20.3)	91 (12.6)	25 (39.7)	152 (28.5)	
Ever had a doctor perform a clinical breast exam(s) (CBE) before breast cancer diagnosis					*<0.001*
No	183 (13.8)	91 (12.6)	4 (6.4)	88 (16.5)	
Yes – last CBE performed within the last year	795 (60.1)	482 (66.5)	35 (55.6)	278 (52.1)	
Yes – last CBE performed more than one year ago	332 (25.1)	146 (20.1)	23 (36.5)	163 (30.5)	
Ever performed breast self-exams (BSEs) before breast cancer diagnosis					*<0.001*
No	283 (21.4)	197 (27.2)	25 (39.7)	61 (11.4)	
Yes – BSEs performed less than once per month	375 (28.4)	184 (25.4)	24 (38.1)	167 (31.3)	
Yes – BSEs performed at least once per month	657 (49.7)	338 (46.6)	14 (22.2)	305 (57.1)	
Ever diagnosed with a benign breast disease before breast cancer diagnosis					*0.042*
No	839 (63.5)	443 (61.1)	42 (66.7)	354 (66.3)	
Yes	476 (36.0)	281 (38.8)	20 (31.8)	175 (32.8)	
Breast tumor clinicopathologic features					
Tumor grade					*<0.001*
Well/moderately differentiated	608 (46.0)	357 (49.2)	36 (57.1)	215 (40.3)	
Poorly differentiated	483 (36.5)	178 (24.6)	22 (34.9)	283 (53.0)	
Unknown	231 (17.5)	190 (26.2)	5 (7.9)	36 (6.7)	

Table 3: Continued.

	Overall (N = 1643) *N (%)*	Screening mammogram (n = 725) *n(%)*	Clinical breast exam (n = 63) *n(%)*	Self-detection (n = 534) *n(%)*	*P*-value
SEER summary stage					*<0.001*
In situ	166 (12.6)	144 (19.9)	2 (3.2)	20 (3.8)	
Localized	718 (54.3)	427 (58.9)	37 (58.7)	254 (47.6)	
Regional/distant	376 (28.4)	115 (15.9)	23 (36.5)	238 (44.6)	
Unstaged	15 (1.1)	5 (0.7)	1 (1.6)	9 (1.7)	
Missing	47 (3.6)	34 (4.7)	0 (0.0)	13 (2.4)	
Tumor size (cm)					*<0.001*
<1.0	509 (38.5)	413 (57.0)	15 (23.8)	81 (15.2)	
1.0-2.0	356 (26.9)	196 (27.0)	22 (34.9)	138 (25.8)	
>2.0	456 (34.5)	115 (15.9)	26 (41.3)	315 (59.0)	
Unknown	1 (0.1)	1 (0.1)	0 (0.0)	0 (0.0)	
Lymph node status					*<0.001*
Negative	814 (61.6)	500 (69.0)	38 (60.3)	276 (51.7)	
Positive	378 (28.6)	119 (16.4)	21 (33.3)	238 (44.6)	
Unknown	130 (9.8)	106 (14.6)	4 (6.4)	20 (3.8)	
Lymphovascular invasion present					*<0.001*
No	856 (64.8)	487 (67.2)	45 (71.4)	324 (60.7)	
Yes	207 (15.7)	59 (8.1)	11 (17.5)	137 (25.7)	
Unknown	259 (19.6)	179 (24.7)	7 (11.1)	73 (13.7)	
Estrogen receptor (ER) status					*<0.001*
ER+	972 (73.5)	573 (79.0)	56 (88.9)	343 (64.2)	
ER-	346 (26.2)	148 (20.4)	7 (11.1)	191 (35.8)	
Unknown	4 (0.3)	4 (0.6)	0 (0.0)	0 (0.0)	
Molecular subtype[a]					*<0.001*
ER+/PR+/HER2-	745 (56.4)	431 (59.5)	44 (69.8)	270 (50.6)	
ER+/PR+/HER2+	127 (9.6)	56 (7.7)	10 (15.9)	61 (11.4)	
ER-/PR-/HER2+	78 (5.9)	33 (4.6)	1 (1.6)	44 (8.2)	
ER-/PR-/HER2-	234 (17.7)	92 (12.7)	6 (9.5)	136 (25.5)	
Unknown	138 (10.4)	113 (15.6)	2 (3.2)	23 (4.3)	

[a]Molecular subtypes were classified using surrogate classifications, based on immunohistochemistry expression of ER and PR, and overexpression of HER2 (by immunohistochemistry and/or fluorescence in situ hybridization) as reported in pathology records.

Table 4: Age-adjusted associations of sociodemographic, reproductive characteristics and medical history with initial mode of breast cancer detection among Black women aged 40-75 years.

	Clinical breast exam compared to screening mammogram Age-adjusted OR (95% CI)	Self-detection compared to screening mammogram Age-adjusted OR (95% CI)
Marital status[a]		
Married or living as married	1.00 (Referent)	1.00 (Referent)
Unmarried	1.25 (0.70, 2.24)	1.27 (0.99, 1.63)
Education		
High school/vocational/technical graduate or below	0.97 (0.5, 1.86)	1.10 (0.82, 1.46)
Some post-secondary education	0.93 (0.43, 2.01)	1.31 (0.95, 1.81)
College graduate or above	1.00 (Referent)	1.00 (Referent)
Annual household income[b]		
<$70,000	1.37 (0.73, 2.58)	*1.46 (1.12, 1.91)*
≥$70,000	1.00 (Referent)	1.00 (Referent)
Primary health insurance at diagnosis[c]		
Private	1.00 (Referent)	1.00 (Referent)
Medicaid	1.30 (0.52, 3.26)	1.14 (0.75, 1.74)
Medicare	1.47 (0.62, 3.49)	0.78 (0.51, 1.18)
Uninsured	1.14 (0.38, 3.37)	*2.16 (1.44, 3.25)*
Other	0.27 (0.04, 2.04)	0.76 (0.45, 1.27)
Menopausal Status		
Premenopausal	*2.68 (1.07, 6.71)*	0.98 (0.66, 1.48)
Postmenopausal	1.00 (Referent)	1.00 (Referent)
Age at menarche (years)		
<12	*0.41 (0.17, 0.99)*	0.90 (0.65, 1.25)
12-13	0.87 (0.45, 1.66)	0.90 (0.67, 1.22)
>13	1.00 (Referent)	1.00 (Referent)
Parity		
Nulliparous	1.00 (Referent)	1.00 (Referent)
1-2 children	1.59 (0.59, 4.25)	0.88 (0.63, 1.24)
≥3 children	2.42 (0.88, 6.63)	1.10 (0.77, 1.59)
Age at first birth (years)		
Nulliparous	1.00 (Referent)	1.00 (Referent)
<25	1.84 (0.69, 4.91)	1.06 (0.75, 1.48)
25-30	2.20 (0.75, 6.46)	0.93 (0.62, 1.41)
>30	1.59 (0.49, 5.19)	0.69 (0.44, 1.09)
History of breastfeeding		
Nulliparous	1.00 (Referent)	1.00 (Referent)
No	2.29 (0.86, 6.08)	1.03 (0.73, 1.46)
Yes	1.54 (0.55, 4.28)	0.90 (0.63, 1.28)
History of oral contraceptive use		
No	1.00 (Referent)	1.00 (Referent)
Yes	0.62 (0.34, 1.12)	0.89 (0.68, 1.17)
History of hormone therapy use		
No	1.00 (Referent)	1.00 (Referent)
Yes	0.52 (0.21, 1.28)	*0.65 (0.46, 0.92)*

Table 4: Continued.

	Clinical breast exam compared to screening mammogram Age-adjusted OR (95% CI)	Self-detection compared to screening mammogram Age-adjusted OR (95% CI)
Family history of breast cancer		
No	1.00 (Referent)	1.00 (Referent)
Yes	1.05 (0.51, 2.16)	0.96 (0.7, 1.31)
Body mass index (kg/m^2)		
<25.0	1.2 (0.52, 2.78)	*1.56 (1.09, 2.22)*
25.0-29.99	0.69 (0.31, 1.51)	0.79 (0.57, 1.09)
30.0-34.99	1.01 (0.48, 2.16)	0.72 (0.51, 1.02)
≥35.0	1.00 (Referent)	1.00 (Referent)

[a]The unmarried category is composed of those who reported being single/never married, separated, divorced, or widowed.

[b]$70,000 is the median income among households in New Jersey and was used as the cut-point to dichotomize the income variable.

[c]The "other" insurance category includes forms of insurance reported by WCHS participants that did not fall into one of the four main categories above and included the following types of insurance coverage: school-provided, national government-provided [foreign country], spousal insurance, health center care, charity care, health savings account, and Veterans Affairs coverage.

≥35.0 kg/m^2) were associated with increased odds of self-detection compared to detection by mammogram. Having a history of HT use (vs. never use: OR 0.65, 95% CI: 0.46, 0.92) was associated with lower odds of self-detection compared to detection through screening mammogram.

As shown in Table 5, ever receiving a routine screening mammogram prior to breast cancer diagnosis (vs. never: OR 0.24, 95% CI: 0.11, 0.55) and performance of BSEs before diagnosis (at least monthly vs. no BSE performance) were associated with lower odds of having CBE as the initial mode of breast cancer detection (OR 0.37, 95% CI: 0.18, 0.78) relative to screening mammogram. Performance of CBEs before diagnosis, particularly having performed the last CBE more than a year before diagnosis (vs. no history of CBE) was associated with higher odds of CBE-detection (OR 4.23, 95% CI: 1.22, 14.62) relative to mammogram-detected breast cancer.

Later stage (regional/distant vs. localized), larger size (1.0-2.0 cm, >2.0 cm vs. <1.0 cm), and positive lymph node status (vs. negative status) were all associated with increased odds of CBE as the initial mode of breast cancer detection, relative to screening mammogram. Similarly, higher tumor grade (poorly differentiated vs. well/moderately differentiated), later stage, larger size, positive lymph node status, presence of lymphovascular invasion (vs. absence), ER- status (vs. ER+), and more aggressive breast cancer subtypes (namely ER-/PR-/HER2+ and ER-/PR-/HER2- vs. ER+/PR+/HER-) were associated with increased odds of breast cancer self-detection compared to detection through mammography.

3.5. Multivariable Regression Analysis of Factors Associated with Mode of Breast Cancer Detection. Results from the multivariable models were relatively consistent with those of the age-adjusted models (Table 6). After adjusting for all covariates, having a routine screening mammogram before breast cancer diagnosis (vs. never having a mammogram) was associated with 80% lower odds of CBE-detected breast cancer (OR 0.20, 95% CI: 0.07, 0.54) relative to mammogram-detected breast cancer. Performance of CBEs more than one year prior to breast cancer diagnosis (vs. no past performance of CBEs) was associated with more than 10-fold the odds of CBE-detected breast cancer (OR 11.04, 95% CI: 2.24, 54.55), while performing BSEs at least once per month (vs. no history of BSEs) was associated with almost 70% lower odds of CBE-detected breast cancer relative to mammogram-detected breast cancer (OR 0.20, 95% CI: 0.07, 0.54).

Women with a normal BMI had increased odds of self-detection relative to detection by mammogram (<25.0 kg/m^2 vs. ≥35.0 kg/m^2: OR 2.46, 95% CI: 1.52, 3.98). In contrast to the relationship with CBE-detection, having a history of performing BSEs (vs. never performing BSEs) was associated with 4-fold (BSEs performed less than once per month: OR 4.08, 95% CI: 2.45, 6.78) to 5-fold (BSEs performed at least once per month: OR 4.99, 95% CI: 3.13, 7.97) the odds of self-detection compared to detection by mammogram. Larger tumors were also associated with increased odds of self-detection (1.0-2.0 vs. <1.0 cm: OR 2.87, 1.81, 4.54; >2.0 vs. <1.0 cm: OR 6.87, 95% CI: 3.54, 13.34) compared to mammogram-detected.

In the sensitivity analysis we found that among women age 50-75, regional/distant SEER stage was associated with more than a 14-fold risk of CBE-detection relative to detection through mammography and history of mammography receipt was associated with lower odds of self-detection relative to mammography (Supplementary Table 1). These associations were not found in the primary analysis that included women age 40-75. In the age-stratified models (Supplementary Tables 2–4), we found that among women age 40-49, BMI <25.0 kg/m^2 and 25.0-29.99 kg/m^2 were associated with increased odds of self-detection relative to detection through screening mammogram (vs. BMI ≥35.0 kg/m^2: OR 8.02, 95% C: 2.77, 23.21 and OR 3.12, 95% CI: 1.24, 7.88, respectively). Having a history of performing BSEs at least once per month before breast cancer diagnosis was associated with almost 5-fold increased odds of self-detection (OR 4.88, 95% CI: 2.00, 11.89); tumor size >2.0 cm (OR 10.57, 95% CI: 2.12, 52.71) and positive lymph node status (OR 5.41, 95% CI: 1.45, 20.15) were associated with increased odds of self-detection compared to detection through screening mammogram. These findings remained relatively consistent among women age 50-59 years, while among women in the 60-75 years age group, only performance of BSEs before breast cancer diagnosis and larger tumor size were found to be associated with increased odds of self-detection relative to detection by screening mammogram.

4. Discussion

Among Black women age 40-75 years, the odds of reporting CBE as the initial mode of breast cancer detection were independently associated with routine breast care characteristics prior to diagnosis (past history of routine screening mammography, performance of a CBE in the year before diagnosis, and having a history of performing BSEs monthly in the year before diagnosis) relative to the odds of reporting screening mammogram as the initial mode of detection. The odds of reporting self-detection as the initial mode of breast cancer were independently associated with lower BMI, history of HT use, having a history of CBEs and BSEs before diagnosis, and larger tumor size, relative to the odds of reporting screening mammogram as the initial mode of detection. Although age was an important confounder of the associations observed, the strong magnitudes of associations observed for breast care characteristics prior to diagnosis and larger tumor size with mode of breast cancer detection remained largely consistent, irrespective of age group (40-49, 50-59, or 60-75 years). The finding that larger tumors were associated with increased odds of CBE-detection and self-detection was not surprising and simply demonstrates that larger tumors are more easily palpable than smaller ones, and therefore can more easily come to the attention of a provider (upon performing a CBE) or the patient (through routinely performing BSEs). The finding that BMI <25 kg/m^2 was associated with increased odds of self-detection supports prior literature showing that overweight/obese women might

Table 5: Age-adjusted associations of breast care characteristics prior to breast cancer diagnosis and tumor clinicopathologic characteristics with initial mode of breast cancer detection among Black women aged 40-75 years.

	Clinical breast exam compared to screening mammogram Age-adjusted OR (95% CI)	Self-detection compared to screening mammogram Age-adjusted OR (95% CI)
Breast care characteristics prior to breast cancer diagnosis		
Ever received a doctor's recommendation for a mammogram		
No	1.00 (Referent)	1.00 (Referent)
Yes	0.70 (0.34, 1.47)	0.73 (0.53, 1.02)
Ever had a routine screening mammogram before breast cancer diagnosis		
No	1.00 (Referent)	1.00 (Referent)
Yes	*0.24 (0.11, 0.55)*	*0.38 (0.24, 0.60)*
Interval between breast cancer diagnosis and most recent routine screening mammogram		
<1 year	1.00 (Referent)	1.00 (Referent)
1 year	0.84 (0.24, 3.00)	0.71 (0.45, 1.12)
>1 year	1.99 (0.56, 7.03)	1.59 (1.00, 2.54)
Ever had a doctor perform a clinical breast exam(s) (CBE) before diagnosis		
No	1.00 (Referent)	1.00 (Referent)
Yes – last CBE performed within the last year	1.47 (0.43, 4.99)	*0.43 (0.30, 0.61)*
Yes – last CBE performed more than one year ago	*4.23 (1.22, 14.62)*	1.13 (0.76, 1.67)
Ever performed breast self-exams (BSEs) before breast cancer diagnosis		
No	1.00 (Referent)	1.00 (Referent)
Yes – BSEs performed less than once per month	1.04 (0.54, 2.02)	*3.06 (2.06, 4.54)*
Yes – BSEs performed at least once per month	*0.37 (0.18, 0.78)*	*3.42 (2.38, 4.93)*
Ever diagnosed with a benign breast disease before breast cancer diagnosis		
No	1.00 (Referent)	1.00 (Referent)
Yes	0.84 (0.46, 1.55)	0.90 (0.7, 1.17)

Table 5: Continued.

	Clinical breast exam compared to screening mammogram Age-adjusted OR (95% CI)	Self-detection compared to screening mammogram Age-adjusted OR (95% CI)
Breast tumor clinicopathologic features		
Tumor grade		
Well/moderately differentiated	1.00 (Referent)	1.00 (Referent)
Poorly differentiated	0.95 (0.50, 1.81)	*2.44 (1.86, 3.21)*
SEER summary stage		
In situ	*0.22 (0.05, 0.94)*	*0.22 (0.12, 0.37)*
Localized	1.00 (Referent)	1.00 (Referent)
Regional/distant	*2.93 (1.59, 5.39)*	*3.21 (2.28, 4.47)*
Tumor size (cm)		
<1.0	1.00 (Referent)	1.00 (Referent)
1.0-2.0	*3.54 (1.64, 7.65)*	*4.07 (2.85, 5.81)*
>2.0	*7.26 (3.41, 15.44)*	*14.64 (10.30, 20.80)*
Lymph node status		
Negative	1.00 (Referent)	1.00 (Referent)
Positive	*2.99 (1.62, 5.51)*	*3.43 (2.58, 4.56)*
Lymphovascular invasion present		
No	1.00 (Referent)	1.00 (Referent)
Yes	1.92 (0.88, 4.18)	*3.21 (2.24, 4.59)*
Estrogen receptor (ER) status[a]		
ER+	1.00 (Referent)	1.00 (Referent)
ER-	0.40 (0.16, 1.03)	*2.06 (1.57, 2.70)*
Molecular subtype[b]		
ER+/PR+/HER2-	1.00 (Referent)	1.00 (Referent)
ER+/PR+/HER2+	1.57 (0.69, 3.56)	1.50 (0.98, 2.31)
ER-/PR-/HER2+	0.32 (0.04, 2.43)	*1.87 (1.12, 3.11)*
ER-/PR-/HER2-	0.48 (0.17, 1.39)	*2.17 (1.56, 3.01)*

[a]As shown in Table 3, percent unknown for tumor characteristics in the overall study sample was as follows: tumor grade, 17.5%; SEER summary stage, 3.6%; tumor size (cm), 0.1%; lymph node status, 9.8%; lymphovascular invasion present, 19.6%; ER status, 0.3%; molecular subtype, 10.4%.

[b]Molecular subtypes were classified using surrogate classifications, based on immunohistochemical expression of ER and PR and overexpression or amplification of HER2 (by immunohistochemistry or fluorescence in situ hybridization) as reported in pathology records.

Table 6: Multivariable logistic regression analysis of the factors associated with mode of detection among Black women aged 40-75 years.

Characteristic	Clinical breast exam compared to screening mammogram Multivariable-adjusted OR (95% CI)	Self-detection compared to screening mammogram Multivariable-adjusted OR (95% CI)
Primary health insurance at diagnosis		
Private	1.00 (Referent)	1.00 (Referent)
Medicaid	0.99 (0.31, 3.15)	1.03 (0.59, 1.82)
Medicare	1.84 (0.63, 5.38)	0.83 (0.48, 1.43)
Uninsured	0.58 (0.14, 2.37)	1.38 (0.77, 2.47)
Other	0.31 (0.04, 2.63)	0.92 (0.46, 1.83)
Body mass index (kg/m^2)		
<25.0	1.33 (0.49, 3.62)	*2.46 (1.52, 3.98)*
25.0-29.99	0.79 (0.32, 1.95)	1.07 (0.70, 1.64)
30.0-34.99	1.22 (0.50, 2.97)	0.80 (0.50, 1.26)
≥35.0	1.00 (Referent)	1.00 (Referent)
History of hormone therapy use		
No	1.00 (Referent)	1.00 (Referent)
Yes	0.61 (0.23, 1.63)	*0.62 (0.39, 0.97)*
Ever had a routine screening mammogram before breast cancer diagnosis		
No	1.00 (Referent)	1.00 (Referent)
Yes	*0.20 (0.07, 0.54)*	0.52 (0.27, 1.00)
Ever had a doctor perform a clinical breast exam(s) (CBE) before breast cancer diagnosis		
No	1.00 (Referent)	1.00 (Referent)
Yes – last CBE performed within the last year	4.33 (0.90, 20.83)	*0.48 (0.29, 0.79)*
Yes – last CBE performed more than one year ago	*11.04 (2.24, 54.55)*	1.28 (0.74, 2.20)
Ever performed breast self-exams (BSEs) before breast cancer diagnosis		
No	1.00 (Referent)	1.00 (Referent)
Yes – BSEs performed less than once per month	0.92 (0.42, 2.01)	*4.08 (2.45, 6.78)*
Yes – BSEs performed at least once per month	*0.31 (0.13, 0.74)*	*4.99 (3.13, 7.97)*

Table 6: Continued.

Characteristic	Clinical breast exam compared to screening mammogram Multivariable-adjusted OR (95% CI)	Self-detection compared to screening mammogram Multivariable-adjusted OR (95% CI)
Breast tumor clinicopathologic features		
Tumor grade[a]		
Well/moderately differentiated	1.00 (Referent)	1.00 (Referent)
Poorly differentiated	0.65 (0.28, 1.47)	1.19 (0.80, 1.77)
SEER summary stage		
In situ	0.44 (0.02, 9.20)	0.65 (0.21, 2.05)
Localized	1.00 (Referent)	1.00 (Referent)
Regional/distant	4.39 (0.78, 24.83)	1.98 (0.92, 4.27)
Tumor size (cm)[a]		
<1.0	1.00 (Referent)	1.00 (Referent)
1.0-2.0	2.13 (0.83, 5.44)	*2.92 (1.84, 4.64)*
>2.0	3.17 (0.88, 11.42)	*6.41 (3.30, 12.46)*
Lymph node status[a]		
Negative	1.00 (Referent)	1.00 (Referent)
Positive	1.54 (0.54, 4.34)	1.62 (0.93, 2.81)
Lymphovascular invasion present[a]		
No	1.00 (Referent)	1.00 (Referent)
Yes	1.18 (0.48, 2.87)	1.35 (0.84, 2.18)
Estrogen receptor (ER) status[a]		
ER+	1.00 (Referent)	1.00 (Referent)
ER-	-	1.13 (0.42, 3.07)
Molecular subtype[a,b]		
ER+/PR+/HER2-	1.00 (Referent)	1.00 (Referent)
ER+/PR+/HER2+	1.44 (0.53, 3.91)	1.08 (0.61, 1.91)
ER-/PR-/HER2+	-	1.44 (0.45, 4.63)
ER-/PR-/HER2-	-	1.69 (0.59, 4.82)

Note: odds ratios (ORs) and 95% confidence intervals (Cis) were generated using multivariable models, mutually adjusting for all variables shown in the table as well as for age.

[a]As shown in Table 3, percent unknown for tumor characteristics in the overall study sample was as follows: tumor grade, 17.5%; SEER summary stage, 3.6%; tumor size (cm), 0.1%; lymph node status, 9.8%; lymphovascular invasion present, 19.6%; ER status, 0.3%; molecular subtype, 10.4%.

[b]Molecular subtypes were classified using surrogate classifications, based on immunohistochemical expression of ER and PR, and overexpression or amplification of HER2 (by immunohistochemistry or fluorescence in situ hybridization) as reported in pathology records.

be less health conscious (i.e., less likely to participate in cancer prevention or related activities including as BSE, CBE, and/or mammography) [30–32]. Evidence also suggests that, compared to overweight/obese women, lower amounts of adipose breast tissue among those with BMI <25 kg/m^2 might result in lower breast volume making it easier to detect breast abnormalities through palpation [33, 34]. A notable finding was that while having a routine screening mammogram before breast cancer diagnosis was significantly associated with lower odds of CBE-detected breast cancer, this factor was not significantly associated with odds of self-detection relative to the odds of mammography-detection.

Among the publications that have examined the characteristics associated with mode of breast cancer detection to date [15–25], most have not examined breast care characteristics occurring prior to breast cancer diagnosis as important predictors. The current study showed that among Black women 40-75 years old, for those that reported having a CBE within one year of diagnosis, there were almost 60% lower odds of breast cancer self-detection and among those who reported having a CBE more than one year before diagnosis, the odds of CBE as the initial mode of detection were 11-fold higher relative to screening mammogram. This is important because factors that affect whether Black women regularly experience opportunities for routine breast care services (i.e., receipt of frequent, routine CBEs) may be associated with socioeconomic status (e.g., income and health insurance status) and healthcare utilization factors (e.g., having a primary care provider, regularly having routine physical exams, etc.), as well as other factors that are not fully understood. Women's knowledge and awareness of the importance of breast health, as well as of breast cancer signs and symptoms are crucial, especially given conflicting screening recommendations and evidence showing that mode of detection may be an independent prognostic factor for breast cancer [15, 17–19].

In terms of self-detection, we found that past performance of BSEs was associated with 4-fold to 5-fold higher odds of self-detected breast cancer relative to mammogram-detected. This finding is particularly concerning in light of the lack of clarity regarding the benefits of BSEs and CBEs as suggested by current USPSTF guidelines, although these guidelines do not consider populations at increased risk for advance disease. Future research should explore the possibility that CBE and BSE can be used as an adjunct to routine mammography for earlier detection. Emerging data, from developing countries [35], suggest that BSE and CBE as adjuncts to screening mammography have effectiveness in clinical down staging among African women who present with later stage disease, similar to patterns observed among Black women in the US. The identification of additional integrated strategies to promote earlier detection among groups at increased risk for breast cancer mortality in the US might also include interventions at the health system level (e.g., improving patient-provider communication around the recommendation for screening mammogram, increasing physician's recommendations for screening mammograms across the board, etc.). In the sample of Black women 40-75 years old included in the current study, of the 725 women who reported that their breast cancer was initially detected through screening mammography, 85.5% reported ever receiving a doctor's recommendation for a mammogram and 86.8% reported ever having a screening mammogram before diagnosis. The proportion of women age 40-49 years who reported receiving a doctor's recommendation for a mammogram was lower (78.9%), although 89.1% of women in this age group reported having a routine screening mammogram before diagnosis. Receiving a doctor's recommendation for a mammogram before diagnosis was not significantly associated with mode of initial breast cancer detection in this study, but receipt of a mammogram prior to diagnosis was associated with reduced odds of initial detection through CBE and through self-detection compared to screening mammography. Evidence suggests that physicians' differential recommendation of screening mammography might contribute to differences in mammography receipt [36–38], thereby potentially contributing to variation in initial mode of breast cancer detection as observed herein.

Our consistent findings of breast care characteristics prior to diagnosis and tumor clinicopathologic features with initial mode of breast cancer detection among Black women in the primary analysis, in the sensitivity analysis, and in the age-stratified analysis suggest that these factors are important, irrespective of age at diagnosis. Since Black women are more likely than any other racial/ethnic group to be diagnosed at a younger age with tumors that are of later stage (and typically exhibit other features that denote more aggressive phenotypes) and have increased odds of breast cancer mortality [6, 8, 12, 39, 40], it is imperative that healthcare providers consult with their Black patients on what age to begin breast cancer screening and whether to receive screening mammograms annually or biennially. This is essential if a patient is considered to be at higher than average risk (i.e., having a strong family history of breast cancer or personal history of breast cancer, benign breast disease, or inherited *BRCA1* and/or *BRCA2* mutations). Postponing mammography screening could increase the possibility of self-detection and may result in detection of breast cancers that are more phenotypically aggressive, leading to poorer breast cancer survival rates [16, 17, 22]. Consequently, if routine and timely breast cancer screening is not equitably provided to all women, disparities in breast cancer outcomes will inevitably continue to widen.

This study has some limitations that should be considered. Presence of missing data for some of the breast cancer clinicopathologic characteristics (i.e., tumor grade, lymphovascular invasion presence, and molecular subtype) could have had an impact on our observed risk estimates. Another limitation is the potential for error and misclassification in some self-reported variables (e.g., sociodemographics, menopausal status, age at menarche, parity, age at first birth, history of breastfeeding, history of oral contraceptive use, history of HT use, and family history of breast cancer). In relation to self-reported mode of initial breast cancer diagnosis, we cannot rule out the possibility that women who reported that they routinely perform BSEs prior to their breast cancer diagnosis and/or reported that their breast cancer was first found through BSE could also be regular participants of mammography screening. There is also a strong possibility that patients who self-reported performing

monthly BSEs were not given systematic instruction on how to perform BSEs and they may have responded that they regularly performed these exams, which might be what they considered a socially acceptable response. However, it is unlikely that recall errors or response bias contributed to variation in initial mode of detection, given that we expect women would know how their breast cancers were detected initially and report it accurately. Therefore, the resulting potential misclassification of initial mode of detection is likely to be random, which would actually lead to an underestimation of the true associations. It would be ideal to investigate the possibility of interval cancers being self-detected or CBE-detected between routine screening mammograms, which can be the focus of future analysis involving medical and pathology data for confirmation. Additionally, it is also essential to further understand whether the observed associations are due to inability of detecting some breast cancers earlier due to factors of a more biologic nature, which might lead to larger breast tumors at diagnosis. Conversely, more phenotypically aggressive breast cancers could grow faster and could be more prone to being found through CBE or BSE/self-detection, before a woman accesses mammography screening or shortly after having a mammogram with normal results. For example, triple-negative and HER2-positive tumors are known to occur as interval tumors (those detected within the period between a normal mammography screening and when the next mammogram is due) [41–43]. Despite these limitations, several statistically significant findings were observed.

Some of the strengths of this study include the utilization of a cohort of well-defined, Black breast cancer cases nested within a case-control study with detailed data available through collection and abstraction of medical and pathology records as well as through interviewer-administered questionnaires. An additional strength is that this study is one of the first to analyze predictors of mode of breast cancer detection among a population of Black women with varying socioeconomic/insurance status. By understanding how specific factors are associated with mode of breast cancer detection among minority women who have a demonstrated disadvantage in breast cancer survival outcomes, we can begin to better understand and develop integrated strategies for addressing disparities in breast cancer outcomes.

5. Conclusion

The findings from this study contribute to the limited literature on factors associated with mode of breast cancer detection, specifically among Black women. Our study found that higher odds of breast cancers being CBE-detected or BSE/self-detected among Black women 40-75 years old were associated with breast care characteristics before breast cancer diagnosis and larger tumor size. These findings highlight the need to improve the rates of early breast cancer detection to potentially reduce breast cancer mortality among populations that currently suffer disproportionate mortality, such as Black women. Healthcare providers must continue educating women on the importance of early breast cancer detection, as well as developing and implementing novel, integrated strategies that promote earlier detection, particularly among women who are at increased risk of being diagnosed with breast cancers having more aggressive phenotypes and who have increased risk of breast cancer mortality.

Data Availability

The sociodemographic, reproductive, clinical, mammographic, and tumor characteristics data used to support the findings of this study may be released upon application to and approval from the Women's Circle of Health Study (WCHS) investigative team, who can be contacted via email at wchstudy@cinj.rutgers.edu. WCHS investigators will make data collected through the study publicly available to outside investigators in compliance with National Institutes of Health (NIH) data sharing policies.

Conflicts of Interest

Holly A. Szukis is currently employed by the Janssen Pharmaceutical Companies of Johnson & Johnson and Kim M. Hirshfield is currently employed by Merck. All other authors declare no conflicts of interest regarding the publication of this article.

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Supplementary Materials

Supplementary Table 1: sensitivity analysis among older women: multivariable logistic regression analysis of the factors associated with mode of detection among Black women aged 50-75 years. Supplementary Table 2: multivariable logistic regression analysis of the factors associated with mode of detection among Black women aged 40-49 years. Supplementary Table 3: multivariable logistic regression analysis of the factors associated with mode of detection among Black women aged 50-59 years. Supplementary Table 4: multivariable logistic regression analysis of the factors associated with mode of detection among Black women aged 60-75 years. *(Supplementary Materials)*

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Research Article

TP53 Mutations Promote Immunogenic Activity in Breast Cancer

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Background. Although immunotherapy has recently achieved clinical successes in a variety of cancers, thus far there is no immunotherapeutic strategy for breast cancer (BC). Thus, it is important to discover biomarkers for identifying BC patients responsive to immunotherapy. *TP53* mutations were often associated with worse clinical outcomes in BC whose triple-negative subtype has a high *TP53* mutation rate (approximately 80%). To explore a potentially promising therapeutic option for the *TP53*-mutated BC subtype, we studied the association between *TP53* mutations and immunogenic activity in BC. *Methods*. We compared the enrichment levels of 26 immune signatures that indicated activities of diverse immune cells, functions, and pathways between *TP53*-mutated and *TP53*-wildtype BCs based on two large-scale BC multiomics datasets. Moreover, we explored the molecular cues associated with the differences in immunogenic activity between *TP53*-mutated and *TP53*-wildtype BCs. Furthermore, we performed experimental validation of the findings from bioinformatics analysis. *Results*. Bioinformatics analysis showed that almost all analyzed immune signatures showed significantly higher enrichment levels in *TP53*-mutated BCs than in *TP53*-wildtype BCs. Moreover, *in vitro* experiments confirmed that mutant p53 could increase BC immunogenicity. Both computational and experimental results demonstrated that *TP53* mutations could promote BC immunogenicity *via* regulation of the p53-mediated pathways including cell cycle, apoptosis, Wnt, Jak-STAT, NOD-like receptor, and glycolysis. Furthermore, we found that elevated immune activity was likely associated with a better survival prognosis in *TP53*-mutated BCs, but not necessarily in *TP53*-wildtype BCs. *Conclusions*. *TP53* mutations may promote immunogenic activity in BC, suggesting that the *TP53* mutation status could be a useful biomarker for stratifying BC patients responsive to immunotherapy.

1. Introduction

The tumor suppressor p53 plays an important role in the regulation of cell-cycle, apoptosis, DNA repair, cellular senescence, and autophagy [1]. Accordingly, *TP53* mutations and dysfunction are importantly involved in carcinogenesis due to the disturbance of these biologic processes it functions in. In fact, *TP53* mutations occur in more than half of all human cancer cases [2] and are independent markers of poor prognosis in a variety of cancers [3]. p53 also plays an important role in immune regulation, e.g., the control of immune responses to infection, autoimmunity, and cancer [4]. p53 functions in immunity by induction of apoptosis, removal of apoptotic cells, antiviral defense, induction of type I IFN, enhanced pathogen recognition, cytokine production, and immune checkpoint regulation [4]. Several studies have explored the association of p53 and tumor immune regulation [5–8]. For example, the p53 activation in the tumor

microenvironment (TME) might overcome tumor immune suppression and enhance antitumor immunity [8]. p53 could transactivate many tumor immunosuppressive genes such as *PD-L1*, *VISTA*, and *FOXP3* [5]. p53 functioned in both tumor suppression and anticancer immunosurveillance via regulation of *VISTA* [5].

Recently, cancer immunotherapy has shown successes in treating various cancers [9]. In particular, the blockade of immune checkpoints has achieved rapid clinical successes in multiple cancers, including skin, lung, kidney, bladder, head and neck cancers, lymphoma, and the cancers with deficient DNA mismatch repair (dMMR) [10]. Unfortunately, current immunotherapies are only propitious to a subset of cancer patients [11]. Some molecular biomarkers associated with cancer immunotherapy response have been identified, e.g., tumor mutation burden (TMB) [12], neoantigens [13], dMMR [14], and PD-L1 expression [15]. However, few studies have correlated the *TP53* mutation status with cancer immunotherapy response, although a recent clinical trial (phase II) data showed that patients with mutated-p53 metastatic breast cancer had better overall survival (OS) when treated with the immunooncology viral agent REOLYSIN® in combination with paclitaxel [16].

Breast cancer (BC) is the most common cancer and the second leading cause of cancer death in women [17]. The triple-negative BC (TNBC) is the BC subtype which does not express estrogen receptor (ER) and progesterone receptor (PR) and lacks overexpression of the human epidermal growth factor receptor 2 (HER2) [18]. TNBC has a high *TP53* mutation rate (80% in TNBC versus 33% in general BC) [19] and has a poor prognosis due to its aggressive clinical behavior and lack of response to hormonal or HER2 receptor-targeted therapy. Although there is currently no immunotherapeutic drug clinically used for BC therapy, several studies have indicated that TNBC might be propitious to immunotherapy [20–22].

To explore the association of *TP53* mutations with tumor immunity in BC, we compared the activity of 26 immune signatures between *TP53*-mutated and *TP53*-wildtype BCs based on the Cancer Genome Atlas (TCGA) [23] and METABRIC [24] BC genomic data. We found that these immune signatures exhibited significantly higher activity in *TP53*-mutated BCs than in *TP53*-wildtype BCs. Furthermore, we explored the molecular cues correlated with the differences in immune activities between *TP53*-mutated and *TP53*-wildtype BCs. Finally, we performed experimental validation of the findings from bioinformatics analysis.

2. Methods

2.1. Datasets. We downloaded TCGA BC RNA-Seq gene expression profiles (Level 3), gene somatic mutations (Level 3), somatic copy number alterations (SCNAs) (Level 3), protein expression profiles (Level 3), and clinical data from the genomic data commons data portal (https://portal.gdc.cancer.gov/), and the METABRIC gene expression profiles, gene somatic mutations, SCNAs, and clinical data from cBioPortal (http://www.cbioportal.org). We obtained 26 gene sets representing 26 different immune signatures from several publications, including 15 immune cell types and functions [25], tumor-infiltrating lymphocytes (TILs) [26], proinflammatory [27], parainflammation (PI) [28], cytokine and cytokine receptor (CCR) [29], human leukocyte antigen (HLA) [22], cancer testis (CT) antigen [30], regulatory T (Treg) cells [31], immune checkpoint [31], metastasis-promoting, and metastasis-inhibiting [32] (Supplementary Table S1). The sample sizes of breast cancers are presented in Supplementary Table S2. We performed computational and statistical analyses using R programming (https://www.r-project.org/).

2.2. Comparisons of Gene Expression Levels, Gene-Set Enrichment Levels, and Protein Expression Levels between Two Classes of Samples. We normalized the TCGA BC gene expression values by base-2 log transformation and used the downloaded normalized METABRIC gene expression data. For the TCGA BC protein expression profiles data, we used the downloaded normalized data. We quantified the activity of an immune signature in a sample by the single-sample gene-set enrichment analysis (ssGSEA) ("GSVA" version 1.24.2, R package) score [33, 34] of the gene set representing the immune signature (a higher ssGSEA score indicated a higher activity) (Supplementary Table S3). We compared gene or protein expression levels between two classes of samples using Student's t test and compared immune signature enrichment levels (ssGSEA scores) between two classes of samples using the Mann-Whitney U test. The false discovery rate (FDR) was utilized to adjust for multiple tests by the Benjamini and Hochberg (BH) method [35]. The threshold of FDR < 0.05 was used to identify the statistical significance. The comparisons involving normal tissue were performed only in TCGA since METABRIC had no normal tissue related data available.

2.3. Comparison of the Immune Cell Infiltration Degree between TP53-Mutated and TP53-Wildtype BCs. We evaluated the immune cell infiltration degree in BC using ESTIMATE ("estimate" version 1.0.13, R package) [36]. For each BC sample, ESTIMATE output an immune score that quantified its immune cell infiltration degree based on the BC gene expression data. In addition, we obtained the lymphocyte infiltration percentage data for BC from the TCGA BC clinical data. We compared immune scores or lymphocyte infiltration percentage between *TP53*-mutated and *TP53*-wildtype BCs using the Mann-Whitney U test.

2.4. Gene-Set Enrichment Analysis. We used GSEA [37] to identify differentially expressed KEGG pathways between *TP53*-mutated and *TP53*-widtype BCs with the threshold of FDR < 0.05.

2.5. Comparison of the Proportions of Leukocyte Cell Subsets between TP53-Mutated and TP53-Wildtype BCs. We used CIBERSORT [38] to calculate the proportions of 22 human leukocyte cell subsets and compared the proportions of the leukocyte cell subsets between *TP53*-mutated and *TP53*-wildtype BCs using the Mann-Whitney U test.

2.6. Correlation of Pathway or Protein Activity with Immune Activity in BCs. We obtained the gene-set collections for p53-mediated pathways from KEGG [39] and quantified the pathway activity with the ssGSEA score of the set of genes included in the pathway. To correct for the strong correlation between the p53 pathway and the other p53-mediated pathways, we evaluated the correlation between a pathway activity and an immune activity using the first-order partial correlation method ("ppcor" version 1.1, R package) [40] with the significance level of FDR<0.05. The correlation between a protein and an immune signature was evaluated by the Spearman correlation coefficient ("rho") of the protein expression levels and the immune signature enrichment levels.

2.7. Survival Analyses. We compared overall survival (OS) and disease-free survival (DFS) between two classes of BC patients divided by the *TP53* mutation status (*TP53*-mutated versus *TP53*-wildtype), or the median values of gene expression levels, immune signature enrichment levels, and immune scores. Kaplan-Meier survival curves were used to exhibit the 20-year survival differences between both groups, and the log-rank test was used to evaluate the significance of survival-time differences with a threshold of $P < 0.05$.

2.8. Comparison of Mutation Counts between TP53-Mutated and TP53-Wildtype BCs. We compared mutation counts (defined as total number of somatic point mutations and indels) between *TP53*-mutated and *TP53*-wildtype BCs using the Mann-Whitney U test. This comparison was performed only in TCGA since somatic mutation data in TCGA were generated by whole exome sequencing while in METABRIC they were generated by targeted exome sequencing.

2.9. In Vitro Experiments

2.9.1. Cell Lines and Cell Culture. Human cells from breast cancer, MCF-7 (ER+/HER2-), and natural killer cells NK-92 were from the American Type Culture Collection (ATCC). MCF-7 was cultured in RPMI-1640 (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA). NK92 cells were incubated in α-MEM (GIBCO, USA) with 2 mM L-glutamine, 0.2 mM inositol, 0.02 mM folic acid, 0.01 mM 2-mercaptoethanol, 10 ng/ml IL-2, 12.5% FBS, and 12.5% horse serum (GIBCO, USA). These cells were cultured in a humidified incubator at 37°C and a 5% CO_2 atmosphere and were harvested in logarithmic growth phase.

2.9.2. Cell Transfection. The MCF-7 cells without antibiotic were maintained in the medium with *TP53*-mutated (c.596 G > T, c.818 A > G, and c.925 T > C) virus stock solution and polybrene (5 ug/mL) for 24h. The transfected MCF-7 cells were cultured in a humidified incubator at 37°C and a 5% CO_2 atmosphere for 48h.

2.9.3. Coculture of MCF-7 and NK-92 Cells. The transwell chamber (Corning Inc., Corning, NY, USA) was inserted into a 6-well plate to construct a coculture system. MCF-7 cells were seeded on the 6-well plate at a density of 5×10^4 cells/well, and NK-92 cells were seeded on the membrane (polyethylene terephthalate, pore size, 0.4μm) of the transwell chamber at a density of 5×10^4 cells/chamber. NK-92 and MCF-7 cells were cocultured in a humidified incubator at 37°C and a 5% CO_2 atmosphere for 24h.

2.9.4. Transwell Migration Assay. After coculture of 24h, NK-92 cells were harvested and resuspended in the upper transwell chambers (8-μm pores, Corning), and MCF-7 cells in the lower 24-well plates. Both NK-92 and MCF-7 cells were incubated at 37°C for 24h. The membrane was removed and its upper surface was wiped away with a cotton swab to remove the unmigrated NK-92 cells. The membrane was fixed in neutral formalin and air-dried at a room temperature and was stained with 0.1% crystal violet at 37°C for 30 min. The number of NK-92 cells that migrated to the lower surface of the membrane was counted under light microscope. Each assay was performed in triplicate wells.

2.9.5. EdU Proliferation Assay. After coculture of 24h, an EdU (5-ethynyl-2′-deoxyuridine, Invitrogen, CA, USA) proliferation assay [41] was performed to measure the proliferation ability of NK-92 cells. NK-92 cells were plated in 96-well plates at a density of 2×10^3 cells/well for 24h. The cells were incubated with 10 μM EdU for 24h at 37°C before fixation, permeabilization, and EdU staining according to manufacturer's protocol. The cell nuclei were stained with DAPI (Sigma) at a concentration of 1 μg/ml for 20 min. The proportion of the cells incorporated EdU was determined with fluorescence microscopy. Each assay was performed in triplicate wells.

2.9.6. CCL4 and CCL5 Enzyme-Linked Immunosorbent Assay. After coculture of 24h, supernatants from NK-92 cells were collected and assayed. CCL4 and CCL5 protein levels were evaluated by ELISA, according to manufacturer's protocol (Shanghai Enzyme-Linked Biotechnology Co., Ltd. China). Study samples and standard dilutions of the chemokines/cytokines were assayed in triplicate. The absorbance was read at 450 nm with the correction set to 570 nm by a microplate reader (BioTek, USA).

2.9.7. Reverse Transcription Quantitative PCR (qPCR) Analysis. Z-DEVD-FMK, abemaciclib, and MPP were purchased from Selleck, Haoyuan Chemexpress, and Cayman, respectively. MCF-7 cells were harvested after being treated by drugs (Z-DEVD-FMK, 50 μM, 72h; abemaciclib, 250 nM, 7 days; MPP, 0.1 nM, 72h). The total RNA was isolated by Trizol (Invitrogen, USA) and was reversely transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA). Primer sequences used for qPCR were presented in the Supplementary Table S4. Primers were diluted in nuclease-free water with the Real time PCR Master Mix (SYBR Green) (TOYOBO Co., LTD, JAPAN). Relative copy number was determined by calculating the fold-change difference in the gene of interest relative to β-actin. The qPCR was performed on an ABI 7500 FAST and Applied Biosystems StepOnePlus Real Time PCR machine.

2.9.8. Western Blotting. MCF-7 cells were washed twice with cold PBS and were lysed in SDS buffer (1% SDS, 0.1 M Tris pH 7.4, 10% glycerol) supplemented with protease inhibitors. The protein concentration was determined by Bradford Protein Assay (Bio-Rad). After normalization of the total protein content, samples were resolved by standard SDS-PAGE. After Western blotting transfer, NC membranes (Millipore) were incubated with antibodies ER-alpha (21244-1-AP, Proteintech Group, INC.) and cleaved-caspase 3 (KGYC0004-6, KeyGEN Biotech, China). After 2h incubation with the HRP-labeled secondary antibody (KGAA002-1, KeyGEN Biotech, China), proteins were visualized by enhanced chemiluminescence using a G: BOX chemiXR5 digital imaging system.

2.9.9. Knockdown of p53 with Small Interfering RNA (siRNA). MCF-7 cells were seeded in 6-well plates and grown until they attained 70% confluency. Transfection of siRNA was performed using Lipofectamine 3000 (Invitrogen, CA) according to the manufacturer's instructions. p53 siRNA and control siRNA were synthesized by KeyGEN BioTECH. The sequence of siRNA against p53 was sense ($5'$-$3'$): CCAUCCACUACAACUACAUdTdT, and antisense ($5'$-$3'$): AUGUAGUUGUAGUGGAUGGdTdG.

2.9.10. Statistical Analyses. All experimental data were expressed as mean ± SD and were analyzed by *t* test using Prism 5.0 software (GraphPad). $P < 0.05$ was considered statistically significant.

3. Results

3.1. TP53-Mutated BCs Exhibit Significantly Stronger Immune Signatures than TP53-Wildtype BCs. Strikingly, we found that almost all 26 immune signatures analyzed showed significantly higher enrichment levels in *TP53*-mutated BCs than in *TP53*-wildtype BCs consistently in both TCGA and METABRIC datasets (Mann-Whitney U test, $P<0.05$; Figure 1(a), Supplementary Figure S1A). Moreover, *TP53*-mutated BCs had significantly higher immune scores than *TP53*-wildtype BCs in both datasets (Mann-Whitney U test; $P=1.35*10^{-7}$. $1.75*10^{-34}$ for TCGA and METABRIC, respectively) (Figure 1(b)). On the basis of the TCGA BC pathological slides data, we found that *TP53*-mutated BCs had markedly higher percentages of lymphocyte infiltration compared to *TP53*-wildtype BCs (Mann-Whitney U test, $P=0.01$) (Figure 1(c)). Altogether, these data indicate that *TP53* mutations are associated with elevated immune activity in BC.

Of the 15 immune cell type and function signatures [25], 14 showed significantly higher enrichment levels in *TP53*-mutated BCs than in *TP53*-wildtype BCs (Mann-Whitney U test, FDR<0.05) (Supplementary Table S5). Moreover, numerous marker genes of immune cells and function had significantly higher expression levels in *TP53*-mutated BCs than in *TP53*-wildtype BCs, e.g., *CD8A* (CD8+ T cell), *B2M*, *HLA-A*, and *TAP1* (MHC Class I), and *GZMA* and *PRF1* (cytolytic activity) (Supplementary Table S5). The TILs signature composed of 122 genes [26] showed significantly higher enrichment levels in *TP53*-mutated BCs than in *TP53*-wildtype BCs (Mann-Whitney U test; $P=2.84*10^{-8}$, $4.22*10^{-33}$ for TCGA and METABRIC, respectively) (Figure 1(a), Supplementary Figure S1B). Moreover, 112 (92%) TIL signature genes were more highly expressed in *TP53*-mutated BCs than in *TP53*-wildtype BCs in at least 1 dataset (88 in both datasets) (Supplementary Table S6; Figure S1C). Altogether, these data indicate that *TP53*-mutated BCs have higher degree of immune cell infiltration than *TP53*-wildtype BCs.

Cytokines are important constituents of the immune system and play crucial roles in the immune regulation of cancer [42]. The enrichment levels of the CCR signature [29] were significantly higher in *TP53*-mutated BCs than in *TP53*-wildtype BCs in both datasets (Mann-Whitney U test; $P=1.9*10^{-10}$, $5.32*10^{-39}$ for TCGA and METABRIC, respectively) (Figure 1(a); Supplementary Figure S1D; Table S7). Of the 261 CCR genes, 158 (61%) were more highly expressed in *TP53*-mutated BCs and 230 (88%) showed more frequent SCNAs in *TP53*-mutated BCs compared to *TP53*-wildtype BCs (Fisher's exact test, FDR < 0.05; Figure 2(a)). These data suggest that *TP53* mutations are associated with higher cytokine activity in BC.

The proinflammatory signature was more enriched in *TP53*-mutated BCs than in *TP53*-wildtype BCs in both datasets (Mann-Whitney U test; $P=2.07*10^{-17}$, $1.81*10^{-61}$ for TCGA and METABRIC, respectively). Strikingly, all 16 proinflammatory genes [27] were upregulated in *TP53*-mutated BCs relative to *TP53*-wildtype BCs in at least 1 dataset (13 in both datasets) (Supplementary Figure S1E, S1F; Table S8). Remarkably, *STAT1* (signal transducer and activator of transcription 1) was upregulated in *TP53*-mutated BCs compared to both *TP53*-wildtype BCs and normal tissue. This gene has been shown to interact with p53 [43] and enhance immunosuppression in BC [44]. Another proinflammatory gene *GZMB* (granzyme B) was upregulated in *TP53*-mutated BCs compared to both *TP53*-wildtype BCs and normal tissue. This gene and *GZMA* (one of the cytolytic activity marker genes upregulated in *TP53*-mutated BCs) are associated with immune cytolytic activity as their protein products are mainly secreted by NK cells and cytotoxic T lymphocytes [25]. These data suggest that *TP53* mutations may promote inflammatory and immune cytolytic activities in BC. In addition, we found that another inflammatory signature parainflammation (PI) [28], a low-grade inflammatory reaction associated with carcinogenesis [28], was more enriched in *TP53*-mutated BCs than in *TP53*-wildtype BCs (Mann-Whitney U test; $P=1.03*10^{-9}$, $5.03*10^{-37}$ for TCGA and METABRIC, respectively) and was also more enriched in both *TP53*-mutated BCs and *TP53*-wildtype BCs than in normal tissue (Mann-Whitney U test; $P=6.89*10^{-12}$, 0.003 for *TP53*-mutated and *TP53*-wildtype BCs, respectively) (Supplementary Table S8). These observations are in line with a previous study showing that PI significantly correlated with the p53 status in cancer [28].

GSEA [37] identified significantly upregulated pathways in *TP53*-mutated BCs compared to *TP53*-wildtype BCs, many of which were immune-related, including natural killer cell mediated cytotoxicity, B cell receptor signaling, antigen

Figure 1: *TP53-mutated breast cancers (BCs) have increased immune activities compared to TP53-wildtype BCs*. (a) Heatmap showing the ssGSEA scores of 26 immune gene-sets in *TP53*-mutated and *TP53*-wildtype BCs (METABRIC). ssGSEA: single-sample gene-set enrichment analysis. TNBC: triple-negative breast cancer. Red color indicates higher enrichment levels (ssGSEA scores) of gene-sets, and blue color indicates lower enrichment levels of gene-sets in the heatmap. *RB1* are more frequently mutated in *TP53*-mutated BCs while *CDH1*, *GATA3*, *MAP3K1*, and *PIK3CA* are more frequently mutated in *TP53*-wildtype BCs (Fisher's exact test, P<0.05). The black vertical lines in the horizontal bars beside gene symbols indicate that the genes are mutated in corresponding samples. The black vertical lines in the horizontal bar beside "TNBC" indicate that the sample is a TNBC. The black vertical lines in the horizontal bars beside "ER", "PR," and "HER2" indicate that the sample is ER-, PR-, or HER2-. (b) *TP53*-mutated BCs have significant higher degree of immune infiltration than *TP53*-wildtype cancers evaluated by ESTIMATE [29]. (c) The TCGA BC pathological slides data show that *TP53*-mutated BCs had markedly higher percent of lymphocyte infiltration than *TP53*-wildtype BCs. *TP53*-mut: *TP53*-mutated BCs. *TP53*-WT: *TP53*-wildtype BCs. It applies to all the other figures.

processing and presentation, intestinal immune network for IgA production, T cell receptor signaling, toll-like receptor signaling, hematopoietic cell lineage, chemokine signaling, primary immunodeficiency, and cytokine-cytokine receptor interaction (Figure 2(b)). In contrast, only the cytokine-cytokine receptor interaction pathway was significantly enriched in *TP53*-wildtype BCs. These results again demonstrate that *TP53* mutations are associated with elevated immune activity in BC.

Furthermore, we compared the proportions of 22 human leukocyte cell subsets that were evaluated by CIBERSORT [38] between *TP53*-mutated and *TP53*-wildtype BCs. We found that *TP53*-mutated BCs harbored higher proportions of activated dendritic cells, M0 macrophages, M1 macrophages, activated T cells CD4 memory, and T cells follicular helper cell subsets (Mann-Whitney U test; FDR<0.05; Figure 2(c)). In contrast, *TP53*-wildtype BCs harbored higher proportions of resting dendritic cells, M2 macrophages,

Figure 2: *TP53-mutated breast cancers (BCs) have higher activity of cytokines, immune pathways, and immune-promoting leukocyte cell subsets than TP53-wildtype BCs*. (a) Cytokine and cytokine receptor (CCR) genes show more frequent somatic copy number alterations (SCNAs) in *TP53*-mutated BCs than in *TP53*-wildtype BCs (Fisher's exact test, FDR<0.05). The outmost circle indicates 23 human chromosomes. The bars in both inner circles (outside and inside) indicate the frequency of SCNAs of CCR genes in *TP53*-mutated and *TP53*-wildtype BCs, respectively. A longer bar indicates a higher frequency of SCNAs. (b) Immune-related KEGG pathways upregulated in *TP53*-mutated BCs relative to *TP53*-wildtype BCs (FDR q-value<0.05). (c) *TP53*-mutated breast cancers (BCs) have significantly different leukocyte cell subset infiltrates estimated by CIBERSORT [30] compared to *TP53*-wildtype BCs.

resting mast cells, monocytes, and resting T cells CD4 memory cell subsets (Mann-Whitney U test; FDR<0.05; Figure 2(c)). This further demonstrates that *TP53* mutations are associated with stronger immune activity in BC. Intriguingly, M1 macrophages that incite inflammation had higher proportions in *TP53*-mutated BCs than in *TP53*-wildtype BCs, while M2 macrophages that repress inflammation and encourage tissue repair had lower proportions in *TP53*-mutated BCs. It suggests that *TP53* mutations may promote inflammatory behavior and inhibit tissue repair in BC, thereby contributing to higher invasiveness of *TP53*-mutated BCs [45].

3.2. TP53 Mutations Are Associated with Elevated HLA Activity in BC. The products of HLA genes MHC proteins play important roles in the regulation of the immune system [46]. We found that most HLA genes showed significantly higher expression levels in *TP53*-mutated BCs than in *TP53*-wildtype BCs (Figure 3(a); Supplementary Table S9; Figure S1G). Moreover, HLA genes were more frequently amplified in *TP53*-mutated BCs compared to *TP53*-wildtype BCs (Figure 3(b)). *TP53*-mutated BCs had lower somatic mutation rates of HLA genes than *TP53*-wildtype BCs in TCGA (Fisher's exact test, P=0.02, OR=0.6), while METABRIC had no somatic mutation data available for HLA genes. These data suggest that *TP53* mutations may promote HLA activity in BC. This finding appears not to be consistent with a previous study showing that p53 increased expression of MHC proteins in cancer [47]. This inconsistency supports the notion that the p53 function is context-dependent and largely depends on the cell type [48, 49].

Gene mutations may yield neoantigens that are associated with antitumor immune response [11]. Although *TP53*-mutated BCs had markedly higher total mutation counts than *TP53*-wildtype BCs in TCGA (Mann-Whitney U test; $P=4.18*10^{-25}$), the numbers of gene mutations yielding predicted HLA-binding peptides [25] showed no significant differences between *TP53*-mutated and *TP53*-wildtype BCs (Mann-Whitney U test; P=0.4). It suggests that TMB or neoantigens may not be the essential factor explaining the differential immune activities between *TP53*-mutated and *TP53*-wildtype BCs.

3.3. Immune Activities Are Associated with Activities of p53-Regulated Pathways in BC. p53 plays important roles in regulating the cancer-associated pathways, e.g., cell cycle, apoptosis, DNA damage repair, autophagy, metabolism, inflammation, epithelial–mesenchymal transition (EMT), angiogenesis, and metastasis [48]. Accordingly, *TP53* mutations often result in the disturbance of the p53-mediated pathways [3]. Indeed, we found that a number of p53-mediated pathways showed significantly differential activity between *TP53*-mutated and *TP53*-wildtype BCs, such as the p53, cell cycle, apoptosis, Jak-STAT, NOD-like receptor, glycolysis, and Wnt pathways showing significantly higher activity in *TP53*-mutated BCs than in *TP53*-wildtype BCs (Mann-Whitney U test; P<0.05). Moreover, these pathways tended to positively correlate with the immune signatures analyzed (Figure 4(a)). These results indicate that the altered immune activity in *TP53*-mutated BCs could be associated with the disturbance of the p53-mediated pathways.

3.4. Identification of Genes and Proteins Differentially Expressed between TP53-Mutated and TP53-Wildtype BCs and Significantly Correlating with Immune Activity in BC. Based on the gene and protein expression data in TCGA, we identified the genes and proteins that were differentially expressed between *TP53*-mutated and *TP53*-wildtype BCs (Student's *t* test; FDR<0.05). Of these, 10 genes (*EGFR*, *CDH3*, *TFRC*, *CCNE1*, *CDK1*, *CDKN2A*, *CHEK1*, *FOXM1*, *NDRG1*, and *STMN1*) and their protein products had significantly higher expression levels in *TP53*-mutated BCs and 8 genes (*ESR1*, *GATA3*, *PGR*, *AR*, *ERBB3*, *BCL2*, *IGF1R*, and *CCND1*) and their protein products had significantly lower expression levels in *TP53*-mutated BCs. We termed the 10 genes and their protein products upregulated in *TP53*-mutated BCs as GPU and the 8 genes and their protein products downregulated in *TP53*-mutated BCs as GPD. Interestingly, GPU had a significant positive expression correlation with almost all 26 immune signatures and immune scores, while GPD had a significant negative expression correlation with them (Spearman correlation, FDR<0.05; Figure 4(b); Supplementary Figures S2A, S2B, S2C). These results showed that the expression of these molecules correlated with elevated or depressed immune activity in BC.

3.5. Association of Immune Activity with Clinical Outcomes in BC. Among 26 immune signatures, 17 and 15 showed a significant correlation with survival (OS and/or DFS) prognosis in *TP53*-mutated and *TP53*-wildtype BCs, respectively (log-rank test; P<0.05) (Figure 5(a); Supplementary Figures S3A, S3B). Strikingly, elevated enrichment of the 17 immune signatures consistently correlated with a more favorable prognosis in *TP53*-mutated BCs. In contrast, 10 and 5 immune signatures were positively and negatively associated with survival in *TP53*-wildtype BCs, respectively. The B cell, cytolytic activity, T cell coinhibition, immune checkpoint, TILs, CCR, HLA, and proinflammatory signatures showed a positive correlation with survival consistently in both *TP53*-mutated and *TP53*-wildtype BCs. However, the CD4+ regulatory T cell signature showed a positive correlation with DFS in *TP53*-mutated BCs while showing a negative correlation in *TP53*-wildtype BCs (Supplementary Figures S3A, S3B; Table S10).

Furthermore, we found numerous immune-related genes whose expression was associated with survival prognosis in *TP53*-mutated and/or *TP53*-wildtype BCs. For example, the immune checkpoint genes *CTLA4*, *PD1*, *PD-L1*, *PD-L2*, and *TIGIT*, and the CD8+ T cell marker gene *CD8A* were positively associated with prognosis in both *TP53*-mutated and *TP53*-wildtype BCs (Figure 5(b); Supplementary Figure S3C). In addition, some genes were positively associated with prognosis exclusively in *TP53*-mutated or *TP53*-wildtype BCs, e.g., *IL10*, *CD247*, *GZMA*, *GZMB*, *CD276*, *CCR4*, and *CCR7* (Supplementary Table S11). Interestingly, some genes had a positive correlation with survival in *TP53*-mutated BCs while having a negative correlation in *TP53*-wildtype BCs,

Figure 3: *TP53-mutated breast cancers (BCs) have more elevated expression of HLA genes than TP53-wildtype BCs*. (a) Heatmap shows that *TP53*-mutated BCs likely more highly express HLA genes than *TP53*-wildtype BCs (METABRIC). (b) HLA genes are more frequently amplified in *TP53*-mutated BCs than in *TP53*-wildtype BCs. The length of the bars in the rose diagram is proportional to the frequency of HLA gene amplification in *TP53*-mutated or *TP53*-wildtype BCs.

Figure 4: *Immune signatures significantly correlate with p53-regulated pathways, genes, and proteins in BC.* (a) Immune signatures likely positively correlate with the p53-mediated pathways that show higher activity in *TP53*-mutated BCs than in *TP53*-wildtype BCs. (b) Immune signatures positively correlate with *EGFR*, *CDH3*, and *TFRC*, and their protein products that are upregulated in *TP53*-mutated BCs, while they negatively correlate with *ESR1*, *GATA3*, and *PCR*, and their protein products that are downregulated in *TP53*-mutated BCs relative to *TP53*-wildtype BCs.

Figure 5: *Immune activities are positively associated with a 20-year survival prognosis in TP53-mutated BCs*. (a) Kaplan-Meier survival curves show that the elevated enrichment of immune signatures is associated with a better 20-year survival in *TP53*-mutated BCs. (b) Kaplan-Meier survival curves show that higher expression levels of immune genes are associated with a better 20-year survival in *TP53*-mutated BCs. The log-rank test P<0.05 indicates the significance of survival-time differences between two classes of patients. HR: hazard ratio; CI: confidence interval.

e.g., *IDO2*, *STAT1*, and *LAG3* (Figure 5(b); Supplementary Table S11; Figure S3C). The mechanism underlying these discrepancies may lie in that *TP53* mutations alter the tumor immune microenvironment (TIM) in BC.

3.6. In Vitro Experiments Validate that TP53 Mutations Promote Immune Activity in BC

3.6.1. TP53 Mutations Increase the Expression of MHC Class I Genes in MCF-7 Cells. We used a pair of isogenic BC cell lines with different p53 status (MCF-7 p53-wildtype versus MCF-7 p53-mutant) and evaluated MHC class I gene expression levels in both cell lines. The MHC Class I genes (*HLA-A*, *HLA-B*, *HLA-C*, and *B2M*) had significantly higher expression levels in p53-mutant MCF-7 cells than in p53-wildtype MCF-7 cells, demonstrated by real-time qPCR (Figure 6(a)). These experimental results verified that *TP53* mutations increased the expression of HLA molecules in BC.

3.6.2. TP53 Mutations Increase the Expression of MHC Class I Genes via Regulation of Apoptosis in BC. p53 plays an important role in regulation of apoptosis [50]. Surprisingly, our bioinformatics analysis showed that *TP53*-mutated BCs had significantly higher activity of the apoptosis pathway than *TP53*-wildtype BCs and that *TP53*-mutated BCs more highly expressed apoptosis-inducing caspases such as *CASP1*, *CASP3*, *CASP4*, and *CASP14* (Figure 6(b)). Furthermore, our experiments verified that caspase-3 expression markedly increased in p53-mutant MCF-7 cells versus p53-wildtype MCF-7 cells (Figure 6(b)). We treated both p53-mutant and p53-wildtype MCF-7 cells with the caspase-3 inhibitor Z-DEVD-FMK and found that the MHC Class I genes had markedly decreased expression in both p53-mutant and p53-wildtype MCF-7 cells (Figure 6(b)). Interestingly, we observed that p53-mutant MCF-7 cells more lowly expressed three out of the four MHC Class I genes than p53-wildtype MCF-7 cells after they were treated with Z-DEVD-FMK. These data indicate that apoptosis may have an appreciable effect on tumor immunity and that *TP53* mutations alter tumor immunity via regulation of apoptosis.

3.6.3. TP53 Mutations Increase the Expression of MHC Class I Genes via Regulation of Cell Cycle in BC. Our bioinformatics analysis showed that *CCND1* (cyclin D1), a regulator of cyclin-dependent kinases, was downregulated in *TP53*-mutated BCs versus *TP53*-wildtype BCs. Furthermore, our experiments verified that *CCND1* had significantly lower mRNA expression levels in p53-mutant MCF-7 cells than in p53-wildtype MCF-7 cells (Figure 6(c)). We treated p53-wildtype MCF-7 cells with the cyclin D1 inhibitor abemaciclib and observed a substantial increase in the expression of MHC Class I genes in MCF-7 cells (Figure 6(c)). Thus, the alteration of the p53-mediated cell cycle pathway may contribute to the differential tumor immunity between *TP53*-mutated and *TP53*-wildtype BCs.

3.6.4. TP53 Mutations Increase the Expression of MHC Class I Genes via Downregulation of Estrogen Receptor Alpha. Our bioinformatics analysis showed that both *ESR1* and its protein product estrogen receptor alpha (ERα) were downregulated in *TP53*-mutated BCs versus *TP53*-wildtype BCs (Supplementary Table S12). This is consistent with previous studies showing that p53 upregulated ERα expression in BC and that *TP53* mutations downregulated ERα expression [51, 52]. Furthermore, our experiments verified that ERα was more lowly expressed in p53-mutant MCF-7 cells than in p53-wildtype MCF-7 cells (Figure 6(d)). We treated p53-wildtype MCF-7 cells with the ERα inhibitor MPP and observed a marked increase in the expression of MHC Class I genes in MCF-7 cells (Figure 6(d)). These results indicate that the downregulation of ERα may contribute to the elevated tumor immunity in *TP53*-mutated BCs.

3.6.5. Mutant p53 Promotes Migration and Proliferation of NK Cells Cocultured with MCF-7 Cells. We used the transwell migration and EdU proliferation assay to observe the migration and proliferation of NK92 cells cocultured with p53-mutant and p53-wildtype MCF-7 cells for 24h, respectively. We found that the number of migrated NK92 cells cocultured with p53-mutant MCF-7 cells far exceeded the number of migrated NK92 cells cocultured with p53-wildtype MCF-7 cells (Figure 7(a)). Moreover, the NK92 cells cocultured with p53-mutant MCF-7 cells showed significantly stronger proliferation ability compared to the NK92 cells cocultured with p53-wildtype MCF-7 cells (Figure 7(b)). Furthermore, we observed that the cytokines CCL4 and CCL5 had markedly higher levels in the serum containing NK92 cells cocultured with p53-mutant MCF-7 cells than in the serum containing NK92 cells cocultured with p53-wildtype MCF-7 cells (Figure 7(c)). These observations verified our computational results that *TP53*-mutated BCs had stronger activities of immune cells including NK cells and more highly expressed a number of CCR genes including *CCL4* and *CCL5* than *TP53*-mutated BCs. These findings are also consistent with previous studies showing that cytokines such as CCL4 could induce NK cells migration [53] and that activated NK cells could secrete cytokines to mediate immune response [54].

4. Discussion

We performed a comprehensive portrait of the association between *TP53* mutations and immune signatures in BC. We found that *TP53*-mutated BCs showed significantly higher levels of immune infiltration and higher activity of various immune cells, function, and pathways than *TP53*-wildtype BCs (Figure 1(a)). *TP53*-mutated BCs had higher proportions of activated immune cell subsets and lower proportions of resting immune cell subsets compared to *TP53*-wildtype BCs within the TME. *TP53*-mutated BCs have significant differences in clinical features compared to *TP53*-wildtype BCs. Typically, *TP53*-mutated BCs contain a higher proportion of ER-, PR-, HER2+, or triple-negative/basal-like BCs. Previous studies have shown that the ER- and HER2+ features were associated with stronger immunogenic activity in BC [22, 55]. Thus, both features may contribute to the higher immune activity in *TP53*-mutated BCs as compared to *TP53*-wildtype BCs. However, when comparing the enrichment

Figure 6: *The expression of MHC Class I genes is significantly upregulated in p53-mutant MCF-7 cells versus p53-wildtype MCF-7 cells and is regulated by the cell cycle, apoptosis, and estrogen receptor (ER) activities.* (a) MHC Class I genes (*HLA-A*, *HLA-B*, *HLA-C*, and *B2M*) have significantly higher mRNA expression levels in p53-mutant MCF-7 cells than in p53-wildtype MCF-7 cells, evident by real-time quantity PCR. (b) Promotion of apoptosis increases the expression of MHC Class I genes, evident by both computational and experimental analyses. (c) Inhibition of cell cycle increases the expression of MHC Class I genes. (d) Inhibition of ER alpha increases the expression of MHC Class I genes. p53-WT: p53-wildtype; p53-mut: p53-mutant; CCND1 i: CCND1 inhibitor; ERa i: ER alpha inhibitor.

Figure 7: *Mutant p53 promotes migration and proliferation of NK cells cocultured with MCF-7 cells.* (a) NK92 cells cocultured with p53-mutant MCF-7 cells show stronger migration ability than NK92 cells cocultured with p53-wildtype MCF-7 cells, evident by transwell migration assay. (b) NK92 cells cocultured with p53-mutant MCF-7 cells show stronger proliferation ability than NK92 cells cocultured with p53-wildtype MCF-7 cells, evident by EdU proliferation assay. (c) Cytokines CCL4 and CCL5 have markedly higher levels in the serum containing NK92 cells cocultured with p53-mutant MCF-7 cells than in the serum containing NK92 cells cocultured with p53-wildtype MCF-7 cells, evident by quantitative enzyme-linked immunosorbent assay (ELISA).

levels of the 26 immune signatures between *TP53*-mutated and *TP53*-wildtype BCs within the ER+ subtype of BC, we obtained similar results that almost all these immune signatures were more enriched in *TP53*-mutated ER+ BCs than in *TP53*-wildtype ER+ BCs (Supplementary Table S13). Similarly, *TP53*-mutated HER2- BCs had significantly higher enrichment levels of immune signatures than *TP53*-wildtype HER2- BCs (Supplementary Table S14). These results indicate that the *TP53* mutation itself is capable of contributing to the elevated immune activity in BC as was further verified by in vitro experiments. Our computational and experimental results suggest that *TP53* mutations may alter immune activity in BC via regulation of the p53-mediated pathways, including cell cycle, apoptosis, Wnt, Jak-STAT, NOD-like receptor, and glycolysis. It should be noted that previous studies have demonstrated that p53 could increase immune activity in various cancers, e.g., colon cancer [47], gastric cancer [56], and lymphoma [8], as appears not to be in line with the present findings. Nevertheless, a number of studies have shown that p53 functions in a context-dependent fashion [8, 48, 49]. Thus, these distinct effects of p53 in regulating tumor immunity could be attributed to the different cellular contexts. Numerous studies have shown that *TP53* mutations may result in not only loss of the wildtype p53 tumor-suppressive function but also gain of oncogenic function of mutant p53 [57]. A recent study showed that *TP53* gain of function mutation could promote inflammatory activity in glioblastoma [58]. To explore whether the elevated immune/inflammatory activity in *TP53*-mutated BC is attributed to *TP53* gain of function mutations, we silenced *TP53* expression in MCF-7 cells by siRNA. Interestingly, we observed a significant increase in the expression levels of MHC Class I genes in p53-knockdown MCF-7 cells compared with p53-wildtype MCF-7 cells (Supplementary Figure S5). This indicates that the elevated immune/inflammatory activity in *TP53*-mutated BC is likely caused by *TP53* loss of function mutations.

One tumor sample may contain a certain percentage of nontumor cells such as normal cells and stromal cells. To exclude the impact of nontumor associated cells on the present results, we selected the BC samples composed of 100% tumor cells based on the TCGA BC pathological slides data. We observed the similar results that almost all 26 immune signatures exhibited significantly higher enrichment levels in the *TP53*-mutated class than in the *TP53*-wildtype class of these samples (Mann-Whitney U test; FDR<0.05) (Supplementary Table S15). Thus, the differential immune activity between *TP53*-mutated and *TP53*-wildtype BCs referred to the actual difference in tumor immunity.

Cancer-testis (CT) antigens are a group of immunogenic proteins overexpressed in many cancers [59]. We found that the CT antigen signature [30] was more active in *TP53*-mutated BCs than in *TP53*-wildtype BCs in both datasets (Mann-Whitney U test; $P=9.52*10^{-35}$, $9.46*10^{-24}$ for TCGA and METABRIC, respectively) (Figure 1(a); Supplementary Figure S4A). Many CT antigen genes were upregulated in *TP53*-mutated BCs and encode the CT antigens that are potential targets for developing cancer vaccines, e.g., *MAGEA*, *NY-ESO-1*, and *PRAME* (Supplementary Figure S4B; Table S16). It suggests that p53 could inhibit the expression of many CT antigens, a finding in line with a prior study [60].

Interestingly, we found that *TP53*-mutated BCs had remarkably higher enrichment levels of Treg signature and immune checkpoint signature [31] than *TP53*-wildtype BCs in both datasets (Mann-Whitney U test; $P<10^{-10}$) (Figure 1(a); Supplementary Figure S4C; Table S17). In particular, numerous notable immune checkpoint genes were upregulated in *TP53*-mutated BCs, including *CTLA4*, *PD1*, *PD-L1*, *PD-L2*, *LAG3*, *IDO1/2*, *BTLA*, *CD80*, *CD86*, *CD27*, and *TIGIT* (Figure 8(a); Supplementary Table S18; Figure S4D). These results suggest that p53 may play a role in inhibiting tumor immunosuppression in BC. Weyden et al. [32] identified 19 genes which function in immune regulation of cancer metastasis, of which 12 promoted tumor metastasis and 7 inhibited tumor metastasis. The enrichment levels of the metastasis-promoting signature were markedly higher in *TP53*-mutated BCs than in *TP53*-wildtype BCs in both datasets (Mann-Whitney U test; $P=3.62*10^{-6}$, 0.003 for TCGA and METABRIC, respectively) (Supplementary Figure 4E). In contrast, the metastasis-inhibiting signature exhibited significantly lower enrichment levels in *TP53*-mutated BCs than in *TP53*-wildtype BCs in TCGA (Mann-Whitney U test; P=0.01) (Supplementary Figure 4E). Notably, *SPNS2* (sphingolipid transporter 2) which most incited tumor metastasis by regulating lymphocyte trafficking [32], had higher expression levels in *TP53*-mutated BCs than in *TP53*-wildtype BCs in TCGA (Student's *t* test; $FDR=1.34*10^{-6}$; *SPNS2* expression data was lacking in METABRIC). These results suggest that *TP53*-mutated BCs are metastasis-prone and that this characteristic may be attributed to the defect in p53 immune regulation of BC and its TME.

The elevated expression of immunosuppressive, proinflammatory, and metastasis-promoting signatures in *TP53*-mutated BCs may promote tumor invasion and lead to a worse prognosis in BC. Indeed, previous studies have shown that p53 mutations were associated with unfavorable clinical outcomes in BC [61, 62]. The METABRIC data also showed that *TP53*-mutated BCs had worse OS and DFS compared to *TP53*-wildtype BCs (Figure 8(b)). Moreover, *TP53*-mutated BCs more highly expressed Ki67 (a marker for cell proliferation) than *TP53*-wildtype BCs (Figure 1(a)), again indicating the higher aggressiveness of *TP53*-mutated BCs. Interestingly, the activities of different immune cell types, function, and pathways, and the immune cell infiltration degree were consistently positively associated with survival prognosis in *TP53*-mutated BCs (Figures 5(a), 5(b), and 8(c)). It is sensible that the elevated enrichment of CD8+ T cell, B cell, NK cell, cytolytic activity, HLA, immune cell infiltrate, TILs, and CCR is associated with favorable clinical outcomes in cancer since these immune signatures can promote anticancer immune response. Furthermore, the observation that the elevated enrichment of Treg, immune checkpoint, proinflammatory, and metastasis-promoting immune signatures was associated with better survival in *TP53*-mutated BCs may be due to the fact that the elevated immunosuppressive activity is likely

Figure 8: *TP53-mutated breast cancers (BCs) more highly express immune checkpoint genes and have a worse 20-year survival than TP53-wildtype BCs and associated with unfavorable survival prognosis in BC, while higher degree of immune cell infiltration is associated with a 20-year better survival prognosis in BC.* (a) A number of important immune checkpoint genes are upregulated in *TP53*-mutated BCs versus *TP53*-wildtype BCs. (b) Kaplan-Meier survival curves show that *TP53*-mutated BCs have a worse 20-year survival prognosis than *TP53*-wildtype BCs. (c) Kaplan-Meier survival curves show that higher degree of immune cell infiltration is associated with a better 20-year survival prognosis in *TP53*-mutated BCs. The log-rank test P<0.05 indicates the significance of survival-time differences between two classes of patients.

to promote chemotherapy sensitivity of *TP53*-mutated BCs [63]. Thus, to achieve successes in immunotherapy of *TP53*-mutated BCs, the effective combination of chemotherapy with immunotherapy may represent a promising direction [64].

Interestingly, compared to *TP53*-wildtype BCs, *TP53*-mutated BCs more highly express a majority of the gene targets for immunotherapy agents that are currently used in the clinic or clinical trials [65] (Supplementary Table S19). It indicates that these immunotherapy agents may be more effective against *TP53*-mutated BCs than *TP53*-wildtype BCs. In fact, several clinical trials [20, 21] have shown that immune checkpoint blockade was effective against TNBC, a BC subtype with a high *TP53* mutation rate.

5. Conclusions

TP53 mutations promote immune activity in BC. This finding suggests that the *TP53* mutation status could be a useful biomarker for stratifying BC patients responsive to immunotherapy.

Data Availability

The TCGA data can be downloaded from the genomic data commons data portal (https://portal.gdc.cancer.gov/), and the METABRIC data can be downloaded from cBioPortal (http://www.cbioportal.org).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary Tables. Table S1: the list of 26 immune signatures and related gene sets. Table S2: sample sizes of breast cancers. Table S3: ssGSEA scores of immune signature in TCGA and METABRIC. Table S4: primer sequences used for real time quantity PCR. Table S5: comparison of the enrichment levels of 15 immune cell types and function signatures between two classes of samples. Table S6: comparison of the enrichment levels of the tumor-infiltrating lymphocytes signature between two classes of samples. Table S7: comparison of the enrichment levels of the cytokine and cytokine receptor signature between two classes of samples. Table S8: comparison of the enrichment levels of the inflammation-promoting and parainflammation (PI) signatures between two classes of samples. Table S9: comparison of the enrichment levels of the HLA signature between two classes of samples. Table S10: comparisons of the ssGSEA scores of immune signatures between *TP53*-mutated and *TP53*-wildtype BCs and their associations with survival prognosis in BC. Table S11: comparisons of the expression levels of immune genes between *TP53*-mutated and *TP53*-wildtype BCs and their associations with survival prognosis in BC. Table S12: comparisons of the expression levels of genes and their protein products between TP53-mutated and TP53-wildtyped BCs. Table S13: comparisons of the enrichment levels of immune signatures between *TP53*-mutated and *TP53*-wildtype BCs within the ER+ subtype of BC. Table S14: comparisons of the enrichment levels of immune signatures between *TP53*-mutated and *TP53*-wildtype BCs within the HER2- subtype of BC. Table S15: comparisons of the enrichment levels of immune signatures between *TP53*-mutated and *TP53*-wildtype BCs within the 100% tumor purity of BC. Table S16: comparisons of the enrichment levels of the cancer-testis signature between two classes of samples. Table S17: comparisons of the enrichment levels of the Treg signature between two classes of samples. Table S18: comparisons of the enrichment levels of the immune checkpoint signature between two classes of samples. Table S19: comparisons of the expression levels of the genes targeted by immunotherapy agents in clinical use or trials or in preclinical development between *TP53*-mutated and *TP53*-wildtype BCs. *Supplementary Figures Legends*. Figure S1: *TP53-mutated breast cancers (BCs) have increased immune activity compared to TP53-wildtype BCs*. A: heatmap shows the ssGSEA scores of 26 immune signatures in *TP53*-mutated and in *TP53*-wildtype BCs (TCGA). ssGSEA: single-sample gene-set enrichment analysis; TNBC: triple-negative breast cancer. *RB1* is more frequently mutated in *TP53*-mutated BCs while *CDH1*, *GATA3*, *MAP3K1*, and *PIK3CA* are more frequently mutated in *TP53*-wildtype BCs (Fisher's exact test; $P<0.05$). The black vertical lines in the horizontal bars beside gene symbols indicate that the genes are mutated in corresponding samples. The black vertical lines in the horizontal bar beside "TNBC" indicate that the samples are TNBC. The black vertical lines in the bars beside "ER", "PR," and "HER2" indicate that the samples are ER-, PR-, or HER2-. B: *TP53*-mutated BCs have higher levels of tumor-infiltrating lymphocytes (TILs) infiltration than *TP53*-wildtype BCs (Mann-Whitney U test; $p<0.001$). C: heatmaps show that *TP53*-mutated BCs likely more highly express TILs genes than *TP53*-wildtype BCs. D: TP53-mutated BCs have higher enrichment levels of the cytokine and cytokine receptor (CCR) signature than *TP53*-wildtype BCs. E: heatmaps show that *TP53*-mutated BCs likely more highly express proinflammatory and parainflammation (PI) gene signatures than *TP53*-wildtype BCs. F: most of the proinflammatory genes are upregulated in *TP53*-mutated BCs relative to *TP53*-wildtype BCs. G: heatmaps show that *TP53*-mutated BCs likely more highly express HLA genes than *TP53*-wildtype BCs in TCGA. $*$, $P<0.05$. $**$, $P<0.01$. $***$, $P< 0.001$. It also applies to the following figures. Figure S2: *the genes and their protein products have a significant expression correlation with immune activities in BC that are differentially expressed between TP53-mutated and TP53-wildtype breast cancers (BCs)*. A: the genes *TFRC*, *CDH3*, and *EGFR* show a positive expression correlation with immune activities in BC. B: the genes *GATA3*, *IGF1R*, *PGR*,

ESR1, *ERBB3*, *BCL2*, and *AR* show a negative expression correlation with immune activities in BC. C: *AR*, *ERBB3*, *BCL2*, and *IGF1R*, and their protein products consistently show a negative expression correlation with immune scores in BC. Figure S3: *association of immune signatures with clinical outcomes in breast cancers (BCs)*. A: Kaplan-Meier survival curves show that the upregulation of immune signatures is consistently associated with better survival in *TP53*-mutated BCs. B: Kaplan-Meier survival curves show that the upregulation of immune signatures is associated with better or worse survival in *TP53*-wildtype BCs. C: the genes *IDO2*, *STAT1*, and *LAG3* have a negative expression correlation with survival prognosis in *TP53*-wildtype BCs. Figure S4: *TP53-mutated breast cancers (BCs) have higher expression levels of cancer-testis (CT) antigens, Treg, immune checkpoint, metastasis-promoting, and metastasis-inhibiting immune signatures than TP53-wildtype BCs*. A: a number of genes encoding the CT antigens that are potential targets for developing cancer vaccines are upregulated in *TP53*-mutated BCs relative to *TP53*-wildtype BCs. B: TP53-mutated BCs have higher enrichment levels of the CT antigen signature than *TP53*-wildtype BCs. C: *TP53*-mutated BCs have higher enrichment levels of the Treg and immune checkpoint signatures than *TP53*-wildtype BCs. D: a number of important immune checkpoint genes are more highly expressed in *TP53*-mutated BCs than in *TP53*-wildtype BCs in TCGA. Treg: regulatory T cell. E: the metastasis-promoting and the metastasis-inhibiting immune signatures are significantly upregulated and downregulated in *TP53*-mutated BCs versus *TP53*-wildtype BCs. Figure S5: *knockdown of p53 by siRNA significantly increases the expression of MHC Class I genes in MCF-7 cells*. *(Supplementary Materials)*

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Research Article

Imaging Biomarkers as Predictors for Breast Cancer Death

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Background. To differentiate the risk of breast cancer death in a longitudinal cohort using imaging biomarkers of tumor extent and biology, specifically, the mammographic appearance, basal phenotype, histologic tumor distribution, and conventional tumor attributes. *Methods*. Using a prospective cohort study design, 498 invasive breast cancer patients diagnosed between 1996 and 1998 were used as the test cohort to assess the independent effects of the imaging biomarkers and other predictors on the risk of breast cancer death. External validation was performed with a cohort of 848 patients diagnosed between 2006 and 2010. *Results*. Mammographic tumor appearance was an independent predictor of risk of breast cancer death (P=0.0003) when conventional tumor attributes and treatment modalities were controlled. The casting type calcifications and architectural distortion were associated with 3.13-fold and 3.19-fold risks of breast cancer death, respectively. The basal phenotype independently conferred a 2.68-fold risk compared with nonbasal phenotype. The observed deaths did not differ significantly from expected deaths in the validation cohort. The application of imaging biomarkers together with other predictors classified twelve categories of risk for breast cancer death. *Conclusion*. Combining imaging biomarkers such as the mammographic appearance of the tumor with the histopathologic distribution and basal phenotype, accurately predicted long-term risk of breast cancer death. The information may be relevant for determining the need for molecular testing, planning treatment, and determining the most appropriate clinical surveillance schedule for breast cancer patients.

1. Introduction

The first generation of breast cancer prognostic indicators, specifically tumor size, histologic malignancy grade, and spread to lymph nodes have long influenced treatment regimens [1–4]. However, these conventional tumor attributes may not be sufficient for predicting the prognosis of breast cancer after the era of early detection via the wide spread use of mammography and molecular tumor subtypes. The mammographic appearance, which reflects pathological changes of breast anatomy, has been reported to be a reliable, independent imaging biomarker for breast

cancer death [5–9]. Six subtypes have been classified based on the features of tumor mass and calcifications [10]. In addition to making use of radiological morphology, over the past decade molecular markers and gene expression profiling have been considered a new set of prognostic indicators. The first molecular breast cancer biomarkers with predictive value were described by Perou [11] and since then additional markers have been identified [12–16]. The most interesting marker may be the basal phenotype, which reportedly is related to the *BRCA*1 mutations and has been shown to be associated with unfavorable prognosis [16, 17]. The early detectability of breast cancer is further enhanced by assessing the extent of breast tumors determined by large-section histopathology [5, 6, 18–22]. In particular, the distribution of the breast cancer foci, determined by large-section histology, has been advocated to differentiate between breast cancer with a good prognosis (unifocal tumors) and breast cancer with poorer prognosis (multifocal and diffuse breast cancers) [23].

Information on mammographic appearance, tumor phenotype, and histological tumor distribution together with conventional tumor attributes is readily available in countries with organized mammography screening programs, and these features are highly relevant where a significant and growing fraction of patients are diagnosed with small, favorable prognosis tumors. A better understanding of long-term prognostic indicators associated with small tumors is desirable to avoid overtreatment, but also to ensure appropriate treatment in the small percentage of patients with small tumors whose poor prognosis presently is not well predicted by conventional tumor attributes [24]. The tumor's appearance on the mammogram, a low resolution black, and white image of the underlying histopathology can contribute additional useful information for treatment planning when combined with the molecular characteristics of tumors.

The future of individualized medicine depends on continued progress in risk profiling based on new tumor markers and other useful disease features and on identifying the clinical circumstances when various combinations of these data are useful for treatment planning. How to identify the complementary role and relevance of each factor with the others in predicting the risk of breast cancer death is of paramount important. For instance, the clinical outcome of the two most fatal breast cancer subtypes, as determined by radiological morphology (architectural distortion without calcification, and casting type calcifications) is not predictable using existing biomarkers [5, 6]. The highly fatal diffuse breast cancers appearing on the mammogram as architectural distortion are generally moderately differentiated cancers, most of which are ER/PR positive, HER2 negative, with low proliferation index (1-10% Ki67). In cases with casting type calcifications on the mammogram and an associated small invasive cancer, the biomarkers are unfortunately determined only on the small invasive cancer tissue rather than on the neoductgenesis represented by the casting calcifications [5].

The aim of the present study is to explore whether imaging biomarkers such as the mammographic appearance of the tumor, the tumor phenotype with particular emphasis on basal phenotype, and the histologic tumor distribution (unifocal, multifocal, and diffuse) can be combined to differentiate risk groups for breast cancer death in a longitudinal cohort of women diagnosed with breast cancer that was derived from a population-based organized mammography screening program.

2. Materials and Methods

2.1. Study Subjects and Data Collection. We identified 1,346 consecutive patients diagnosed with primary invasive breast cancer at Falun Central Hospital, Sweden, of which 498 were diagnosed between 1996 and 1998 and 848 were diagnosed between 2006 and 2010. A prospective cohort study design was adopted by treating the former series as the test cohort and the latter series as the validation cohort. The predictors considered were based on the mammographic appearance of the tumor, basal phenotype, and the histologic tumor distribution together with the conventional tumor attributes (tumor size, axillary node status, and histologic malignancy grade). Note that as type of surgery and adjuvant therapy are driven by these correlated but complementary features, they are regarded as controlled variables when the respective independent effects of each factor on the survival of breast was assessed. Date and cause of death were obtained from the Swedish National Death Registry.

Breast cancer care at Falun Central Hospital has been coordinated by a multidisciplinary tumor board including radiologists, pathologists, breast cancer surgeons, and oncologists to review the screening mammogram, confirmatory diagnosis by ultrasound, MRI, and other alternative imaging studies, pathological reports based on large-section method, and the choice of treatment modalities. Information on mammographic appearance of the tumor, including tumor distribution and extent, was available to a preoperative tumor board for each case. After excision, with the aid of whole specimen radiograph, the most appropriate sections were obtained to represent the proper extent of the breast lesion. Each case was discussed by the postoperative tumor board, which verified the concordance of radiologic and histologic findings. The three families of predictors were determined as follows.

2.2. Mammographic Appearance of the Tumor. The classification system developed by Tabár was used to assess mammographic tumor appearance [6, 25]. Six mammographic categories were used, each having histologically proven invasive carcinoma: (1) stellate mass with no associated calcifications; (2) circular mass with no associated calcifications; (3) calcifications of crushed stone-like appearance, with or without associated tumor mass; (4) powdery calcifications, with or without associated tumor mass; (5) casting type calcifications, with or without associated tumor mass; and (6) architectural distortion.

2.3. Molecular Classification. Immunohistochemical assessment was made using the methods for molecular phenotypes given by Pekar et al. [26]. Tumors expressing at least one basal (myoepithelial) marker (CK5/6, CK14, or EGFR) were

categorized as basal phenotype tumors [26]. Other markers such as HER-2, ER and PR status were used to compare various classification systems (see supplementary Tables S1-S6).

2.4. Tumor Attributes, Histologic Tumor Distribution, and Treatment Modalities. The details of the determination of these pathological features have been fully described elsewhere [21, 23]. Briefly, a team of four pathologists reviewed all large-format histology slides without having information regarding the mammographic tumor features. The 1996-1998 material was reviewed at a meeting of the four pathologists on our breast team. They analyzed the subgross features on an overhead monitor and the microscopic features in a multiheaded microscope. The pathohistological parameters in the original pathology report were either confirmed or modified following the consensus in the group. The 2006-2010 material was reviewed by three pathologists. Histologic tumor distribution was classified as unifocal, multifocal, or diffuse. Representative tumor areas were identified for taking punch biopsies to determine the phenotype of the tumor with immunohistochemistry. In unifocal carcinomas with heterogeneous histologic appearance, as well as multifocal and diffuse cancers, multiple punch biopsies were obtained. In addition to histologic tumor distribution, conventional tumor attributes (tumor size, node status, and histologic malignancy grade) were also determined from the large-section histology [23]. Tumor size was categorized as a binary variable: 1-14 mm or ≥ 15 mm. Axillary lymph node involvement was classified as positive or negative. Histologic malignancy grade was also determined and classified as well-differentiated, moderately differentiated, or poorly differentiated following the Bloom-Richardson system as modified by Elston and Ellis [27]. Note that these three pathologists in the late period were the same members in the original team between 1996 and 1998. The infrastructure of this department of pathology is very stable with time and the consistency between two cohorts in terms of operational viewpoint on pathology is high. For example, the large-section method for detecting histological tumor distribution (such as unifocal, multifocal, and diffuse) and microarray analysis has been adopted by this team since 1996.

2.5. Statistical Analysis. We assessed the associations between pairs of predictors in the two cohorts combined using the chi-squared test. The relative contribution of each predictor to the risk of breast cancer death was determined based on Cox proportional hazards regression analysis of the first series of 498 patients (diagnosed between 1996 and 1998) who were followed until death or December 31, 2010. Survival time was computed from the date of diagnosis until death or the end of the study. Breast cancer death was treated as an uncensored event, whereas other causes of death were regarded as censored events. The survival curves by mammographic appearance of the tumor, basal phenotype, and histologic tumor distribution were plotted using the life-table method.

In addition to the descriptive analysis indicated above, the present study has two main analyses for deriving informative analytic results. The first analysis is to assess the respective independent effects of each relevant variable on the survival of breast cancer. We estimated the hazard ratios (HRs) for mammographic appearance, basal phenotype, histologic tumor distribution, conventional tumor attributes, type of surgery, and adjuvant therapy. In the multivariable regression model, we treated tumor size, node status, histologic malignancy grade, type of surgery, and adjuvant therapy as established predictors that are retained in the basic model although these variables are correlated with or driven by the imaging biomarkers of interest. After first incorporating the mammographic appearance of the tumor, we observed several issues of collinearity when we added basal phenotype and histologic tumor distribution to assess whether they were prognostic predictors independent of the tumor's mammographic appearance. For example, the stellate, powdery calcifications, and crushed stone-like calcification appearances were rarely seen in combination with the basal phenotype (see below). We therefore combined these three categories in order to produce concise information, since previous results suggested that they all have similar prognosis [10]. Because of the similarity of the prognosis, the clinical treatment and therapies are driven by imaging biomarkers in the same way that certain therapies are only prescribed for specific molecular tumor subtypes (e.g., adjuvant hormone therapy would only be prescribed for hormone sensitive tumors).

The second analysis is focused on producing a new classification of risk groups based on empirical survival estimates before the administration of treatment and adjuvant therapies and the selected variables with reference to the results of the Cox proportional hazards regression model and the cumulative survival curves. We classified the three-dimensional variables (mammographic appearance of the tumor, basal phenotype, and histologic tumor distribution) into 12 risk groups. The 10 yr risk of breast cancer death and the 95% confidence interval directly using the test cohort data are presented. Note that the order of predictors used for the new classification is not based on statistical criteria but pursuant to strong evidence from the chronological order of acquired clinical information, beginning with the finding of mammographic features, the immunochemical test with emphasis on basal phenotype, large-section pathological findings, and tumor size, all of which led to the decision of surgical type and adjuvant therapy.

To validate the proposed model, we estimated regression coefficients (clinical weights) for each group, with adjustment for conventional tumor attributes, type of surgery, and adjuvant therapy, using the Weibull accelerated failure time model [28]. We predicted the risk of breast cancer death by applying the estimated regression coefficients to the validation cohort which was followed until Dec. 31, 2015. As these patients were diagnosed with breast cancer between 2006 and 2010, the regression coefficients estimated from the Weibull accelerated failure time model were adjusted for the baseline hazard rate corresponding to the underlying rate of breast cancer death for the period between 2006 and 2015.

As far as this validation procedure is concerned, we follow the traditional paradigm that divides the entire dataset into the trained dataset and the validation dataset. The only difference is that the decision influencing the selection of each dataset is based on their chronological order with external validation, rather than the division of the entire cohort into the test cohort and the validation cohort with cross-validation, because there was interest in examining whether imaging biomarkers identified in the early period of digital mammography (1996-1998) can be applied to the latest period (2006-2010). The test dataset was used to estimate the weights, namely, the regression coefficients, contributed from each predictor of these emerging imaging biomarkers, tumor phenotypes, and conventional tumor attributes that have been well recognized from numerous previous clinical studies without doing model selection. The effect sizes of these regression coefficients were further applied to the validation dataset to estimate the expected values in comparison with the observed one to check the adequacy of the proposed predicted risk model using a chi-squared test. We also compared the performance of the model with the incorporation of imaging biomarkers with one that only used conventional tumor attributes by using the area under the curve (AUC) of the receiving operating characteristics (ROC) in order to assess the additional performance of these imaging biomarkers, although such a comparison may be conservative as certain imaging biomarkers may be an early indicator of these conventional tumor attributes.

Use of anonymized data for academic purposes was approved by the Regional Ethical Committee Uppsala, Örebro, Sweden (Dnr 2008/081).

3. Results

We had data on 1,346 women diagnosed with invasive breast cancer from 1996 to 1998 (n=498, the test cohort) and 2006 to 2010 (n=848, the validation cohort). The median follow-up time was 12.43 years for women diagnosed between 1996 and 1998 and 6.97 years for women diagnosed between 2006 and 2010. All women are Caucasians. Table 1 shows the demographics, mammographic appearance of the tumor, basal phenotype, histologic tumor distribution, conventional tumor attributes, type of surgery, and adjuvant therapy (radiotherapy, chemotherapy, and Tamoxifen) in the two cohorts.

3.1. Long-Term Breast Cancer Survival by Imaging Biomarkers. The 14-year cumulative survival curves by mammographic appearance of the tumor, basal phenotype, and histologic tumor distribution status are shown in Figures 1(a)–1(c). The survival curves by mammographic appearance of the tumor (Figure 1(a)) clearly illustrate that the prognosis was most favorable for tumors with powdery calcifications (92.9%) and the crushed stone-like type (91.7%), followed by the stellate (80.6%) and the circular types (82.6%); the casting type was associated with poor survival (65.4%), and the survival rate for the architectural distortion type (44.1%) was extremely poor. The difference in the 14-year survival across the six appearance types was statistically significant ($p<0.0001$). As seen in Figure 1(b), 14-year survival was poorer for the basal phenotype compared with the nonbasal phenotype (62.5% versus 84.6%; $p<0.0001$). Fourteen-year survival results differed significantly ($p<0.0001$) by histologic tumor distribution: diffuse type, 63.9%; multifocal type, 71.2%; and unifocal type, 88.8% (Figure 1(c)).

3.2. Associations between Imaging Biomarkers. The associations between mammographic tumor appearance and each of the other predictors, including basal phenotype, histologic tumor distribution, and conventional tumor attributes, are presented in Table 2. We combined three mammographic tumor appearance features (stellate tumors, powdery calcifications, and crushed stone-like calcifications on the mammogram) as noted above. Breast tumors with a circular appearance on mammography were more likely to have the basal phenotype (22.0%) compared with tumors showing the other appearance types (architectural distortion, 16.7%; casting-type calcifications 11.9%; and stellate tumors, powdery calcifications, or crushed stone-like calcifications on the mammogram 3.1%).

The association between mammographic tumor features and the histologic tumor distribution is striking. More than 70% of the casting-type tumors and those with architecture distortion were diffuse and had poor survival. Conversely, most circular and stellate/powdery/crushed stone-like types, which had good survival rates, were unifocal.

The mammographic tumor appearance was also associated with conventional tumor attributes (tumor size, histologic malignancy grade, and positive lymph nodes). Circular and stellate/powdery/crushed stone-like types were associated with smaller, node-negative, and well-differentiated breast tumors. The similar results based on the test cohort alone are specified in Table S1.

The associations between basal phenotype and other predictors using the test cohort are specified in Table S2. Basal phenotype and histologic tumor distribution were not significantly associated. Compared with the nonbasal phenotype, the basal phenotype was associated with larger, poorly differentiated breast tumors, and node-positive tumors. Table S3 presents the relationships between histologic tumor distribution and the other predictors using the test cohort. Large, node-positive, and poorly differentiated breast tumors were observed in both the multifocal and diffuse breast cancers, showing an association between histologic tumor distribution and conventional tumor attributes. Tables S4-S6 show the similar results based on the validation cohort.

3.3. Assessment of Independent Effect. Table 3 gives the adjusted and unadjusted hazard ratios for breast cancer death and 95% confidence intervals (CI), with the estimated results for the three main variables of interest in the usual time order of clinical assessment. In the unadjusted (crude) analysis, the appearance of architectural distortion and the presence of casting type calcifications on the mammogram were strong predictors of breast cancer death, conferring 4.73-fold (95% CI: 2.48–9.05) and 2.33-fold (95% CI:1.17–4.66) increased risks, respectively, compared with the

Table 1: Distribution of age, mammographic appearance, histologic tumor distribution, and conventional tumor attributes in two study cohorts.

	Women diagnosed between 1996-1998		Women diagnosed between 2006-2010	
	Number	Percentage (%)	Number	Percentage (%)
Age, years				
<40	13	2.61	21	2.48
40–49	67	13.45	117	13.80
50–59	113	22.69	169	19.93
60–69	113	22.69	254	29.95
70–79	107	21.49	171	20.17
80+	85	17.07	116	13.68
Mammographic appearance				
Stellate	212	43.89	371	46.55
Circular	161	33.33	235	29.49
Powdery	15	3.11	12	1.51
Casting	32	6.63	46	5.77
Crushed stone-like	40	8.28	94	11.79
Architectural distortion	23	4.76	39	4.89
NK	15		51	
Basal type				
Non-basal phenotype	401	90.11	634	88.80
Basal phenotype	44	9.89	80	11.20
NK	53		134	
Histologic tumor distribution				
Unifocal	299	65.28	458	55.25
Multifocal	115	25.11	301	36.31
Diffuse	44	9.61	70	8.44
NK	40		19	
Tumor size (mm)				
1–9	85	18.52	152	18.51
10–14	92	20.04	192	23.39
15–19	95	20.70	178	21.68
20–29	114	24.84	187	22.78
30–49	58	12.64	88	10.72
50+	15	3.27	24	2.92
NK	39		27	
Histologic malignancy grade				
I/II	341	74.13	627	76.84
III	119	25.87	189	23.16
NK	38		32	
Lymph node involvement				
Negative	331	72.43	513	61.51
Positive	126	27.57	321	38.49
NK	41		14	
Surgery type				
Breast conserving surgery	252	54.55	309	50.32
Mastectomy	210	45.45	305	49.68
NK	36		234	
Radiotherapy				
No	195	39.47	206	34.39
Yes	299	60.53	393	65.61
NK	4		249	

Table 1: Continued.

	Women diagnosed between 1996-1998		Women diagnosed between 2006-2010	
	Number	Percentage (%)	Number	Percentage (%)
Chemotherapy				
No	408	82.59	382	63.88
Yes	86	17.41	216	36.12
NK	4		250	
Hormone therapy				
No	374	75.71	207	34.62
Yes	120	24.29	391	65.38
NK	4		250	

NK, not known.

Table 2: Relationship of mammographic appearance to basal phenotype, histologic tumor distribution, and conventional tumor attributes in both cohorts combined.

	Stellate/Powdery/Crushed stone-like	Circular	Casting	Architectural distortion	NK	χ^2*	P value
Basal phenotype						89.71	<0.0001
Non-basal	619 (96.87)	270 (78.03)	59 (88.06)	45 (83.33)	42		
Basal	20 (3.13)	76 (21.97)	8 (11.94)	9 (16.67)	11		
NK	105	50	11	8	13		
Histologic tumor distribution						817.74	<0.0001
Unifocal	450 (63.11)	260 (68.78)	7 (9.21)	7 (11.48)	33		
Multifocal	257 (36.04)	116 (30.69)	12 (15.79)	9 (14.75)	22		
Diffuse	6 (0.84)	2 (0.53)	57 (75.00)	45 (73.77)	4		
NK	31	18	2	1	7		
Tumor size (mm)						53.96	<0.0001
<15	340 (47.82)	127 (33.78)	31 (42.47)	3 (5.00)	20		
≥15	371 (52.18)	249 (66.22)	42 (57.53)	57 (95.00)	40		
NK	33	20	5	2	6		
Histologic malignancy grade						128.08	<0.0001
I/II	613 (86.46)	227 (60.21)	35 (48.61)	53 (86.89)	40		
III	96 (13.54)	150 (39.79)	37 (51.39)	8 (13.11)	17		
NK	35	19	6	1	9		
Lymph node involvement						22.52	<0.0001
Negative	479 (66.81)	258 (68.07)	45 (59.21)	23 (38.33)	39		
Positive	238 (33.19)	121 (31.93)	31 (40.79)	37 (61.67)	20		
NK	27	17	2	2	7		

∗ Those with unknown mammography feature were not included in the chi-square test. NK, not known.

stellate/powdery/crushed stone-like calcification group. The basal phenotype was associated with a greater risk of breast cancer death than the nonbasal phenotype (HR=3.39; 95% CI: 1.91–6.01). The risk for breast cancer death was 3.80-fold for diffuse tumors and 2.68-fold for multifocal tumors compared with unifocal tumors.

After adjustment for conventional tumor attributes, type of surgery, and adjuvant therapy (Table 3), mammographic tumor appearance remained an independent predictor of breast cancer death (p=0.0003); the presence of casting-type calcifications and architectural distortion on the mammogram was associated with 3.13-fold and 3.19-fold risks, respectively; and the basal phenotype independently conferred 2.68-fold risk compared with the nonbasal phenotype (p=0.0058). Since the effect of diffuse histologic tumor distribution on survival was captured by tumors with casting-type calcifications and architectural distortion, we did not include the effect of the diffuse status in the final multivariable regression model to avoid colinearity. The HR of circular-type basal tumors was clearly affected by the incorporation of tumor size, node status, type of surgery, and adjuvant therapy.

Tables S7-S8 also present the comparison of the results between the trained cohort and the validated cohort. The results of the univariate analysis on the crude hazard ratio (HR) from both cohorts are very similar. Note that the effect sizes of treatment and therapy were affected by the period effect and were included when the predicted model was constructed by using the validation cohort. However, the effect sizes on the results of multivariate analysis based on the validation cohort were attenuated due to insufficient

Table 3: Unadjusted (crude HR) and adjusted hazard ratios (aHR) for the effects of mammographic appearance, basal phenotype, histologic tumor distribution, conventional tumor attributes, surgery, and adjuvant therapy on the risk for breast cancer death.

Characteristic	No. of alive or OCD (%)	No. of BCD (%)	Crude HR (95%CI)	aHR (95%CI)
Mammographic appearance				
Stellate/Powdery/Crushed stone-like	227 (85.02)	40 (14.98)	1.00	1.00
Circular	137 (85.09)	24 (14.91)	1.09 (0.66, 1.80)	0.82 (0.48, 1.39)
Casting	22 (68.75)	10 (31.25)	2.33 (1.17, 4.66)	3.13 (1.46, 6.70)
Architectural distortion	11 (47.83)	12 (52.17)	4.73 (2.48, 9.05)	3.19 (1.55, 6.56)
Basal phenotype				
Non-basal	348 (86.78)	53 (13.22)	1.00	1.00
Basal	29 (65.91)	15 (34.09)	3.39 (1.91, 6.01)	2.68 (1.33, 5.39)
Histologic tumor distribution				
Unifocal	269 (89.97)	30 (10.03)	1.00	1.00
Multifocal	87 (75.65)	28 (24.35)	2.68 (1.60, 4.49)	1.62 (0.95, 2.76)
Diffuse	30 (68.18)	14 (31.82)	3.80 (2.01, 7.17)	–
Tumor size (mm)				
<15	165 (93.22)	12 (6.78)	1.00	1.00
≥15	222 (78.72)	60 (21.28)	3.77 (2.03, 7.02)	1.01† (1.00, 1.02)
Histologic malignancy grade				
I/II	298 (87.39)	43 (12.61)	1.00	1.00
III	89 (74.79)	30 (25.21)	2.28 (1.43, 3.63)	1.10 (0.65, 1.86)
Lymph node involvement				
Negative	298 (90.03)	33 (9.97)	1.00	1.00
Positive	86 (68.25)	40 (31.75)	3.90 (2.46, 6.19)	3.04 (1.76, 5.27)
Surgery type				
Breast conserving surgery	251 (87.15)	37 (12.85)	1.00	1.00
Mastectomy	155 (73.81)	55 (26.19)	2.39 (1.58, 3.63)	1.10 (0.63, 1.91)
Radiotherapy				
No	153 (78.46)	42 (21.54)	1.00	1.00
Yes	250 (83.61)	49 (16.39)	0.59 (0.39, 0.89)	0.59 (0.36, 0.97)
Chemotherapy				
No	342 (83.82)	66 (16.18)	1.00	1.00
Yes	61 (70.93)	25 (29.07)	1.74 (1.10, 2.75)	0.64 (0.36, 1.13)
Hormone therapy				
No	311 (83.16)	63 (16.84)	1.00	1.00
Yes	92 (76.67)	28 (23.33)	1.52 (0.97, 2.37)	1.15 (0.71, 1.87)

† size was used as a continuous variable.
OCD, other causes of death; BCD, breast cancer death; CI, confidence interval.

statistical power as the follow-up time of the validation cohort is too short to have sufficient numbers of breast cancer deaths. The validation dataset is therefore tailored for validation rather than training parameters.

3.4. New Classification of Risk Group. By combining mammographic tumor appearance, basal phenotype, tumor size, and histologic tumor distribution, 12 classification categories of risk profiles for breast cancer death were created (Figure 2). A 10-year risk of breast cancer death of less than 10% is defined as the low-risk group; an 11%-24% risk is the intermediate-risk group; a 25%–40% risk is the high-risk group; and a greater than 40% risk is defined as an extremely high-risk group (Figure 3). Note that Figure 3 also shows how surgical types (conservative surgery and mastectomy) and adjuvant therapies (radiotherapy, chemotherapy, and hormone therapy) were driven by the combination of three-dimensional imaging biomarkers as shown in the box surrounded by the dotted line in Figure 3. For example, the lower the risk of breast cancer death is (such as small circular unifocal tumors with nonbasal phenotype), the more likely the patient will undergo conservative surgery and will be less likely to undergo chemotherapy.

3.5. Validation. The observed breast cancer deaths in the validation cohort in 12 risk categories did not differ significantly from those predicted by the model (p=0.44). The lack of statistical significance suggests that the model is valid. The comparison between the observed and the expected values based on the validation cohort by risk groups has been

Figure 1: Cumulative 14-year survival of invasive breast cancers diagnosed in Falun 1996-1998. (a) According to mammographic tumor features. (b) According to basal phenotype. (c) According to histologic tumor distribution.

given in Table S8. The observed and the expected values in different time periods by risk groups also have been plotted in Figure S1. The discrepancy is very small. However, as numbers of breast cancer are sparse, such a comparison should be taken with great caution. Compared with the model that only included conventional tumor attributes, the AUC of ROC increased from 77% to 84% when the mammographic appearance, basal phenotype, and histological tumor distribution was added to the model.

4. Discussion

Early detection through mammography screening results in a significantly reduced incidence rate of large, node-positive, and poorly differentiated tumors. However, conventional tumor attributes do not readily distinguish the small fraction of cancers that potentially are lethal from the larger fraction that are not. While there has been a growing presence of critical commentary in the literature about overtreatment, a considerable volume of overtreatment occurs among women with small, favorable prognosis cancers. Given the fact that breast cancer treatments often are associated with short and long-term morbidity that can significantly diminish quality of life and functional status, avoiding overly aggressive treatment when it is not needed should be a high priority [29]. Avoiding undertreatment is no less important. Thus, there is a critical need for new indicators to refine the breast cancer risk profiles to choose more optimal treatment modalities.

We combined mammographic tumor appearance, basal phenotype, and histologic tumor distribution to identify a

Figure 2: Cumulative survival of invasive breast cancer diagnosed in Falun 1996-1998, according to mammographic appearance, tumor size, histologic tumor distribution, and basal phenotype.

classification of risk profiles to predict breast cancer death. The risk profiles are based on data from a long-term longitudinal follow-up of 498 invasive breast cancer cases, and this classification was validated using a new independent dataset. These risk profiles can be grouped into 4 categories based on mammographic appearance, basal phenotype, and the combination of histologic tumor distribution and tumor size: the lowest risk group (≤ 10%) consists of stellate unifocal breast tumors <15mm in size, tumors with powdery and crushed stone-like calcifications, circular nonbasal phenotype unifocal tumors, and circular basal phenotype unifocal tumors <15mm in size; the intermediate risk group (11-24%) consists of stellate unifocal tumors ≥15mm in size, breast tumors with stellate multifocal appearance, and circular (basal and nonbasal phenotype) multifocal tumors; the high risk group (25-40%) consists of circular unifocal basil phenotype tumors ≥15 mm in size, and tumors with casting-type calcifications on the mammogram; and the extremely high risk group > 40% presents with architectural distortion without associated calcifications on the mammogram. These risk groups, shown in Figure 3, supplement the role of conventional tumor attributes.

Tumors with casting-type calcifications or architectural distortion without associated calcifications on the mammogram showed the poorest survival regardless of basal phenotype or histologic tumor distribution. However, few basal phenotype tumors were accompanied by calcifications of any kind, with the majority typically being circular masses. Three mammographic tumor appearance types, stellate tumors without calcifications and tumors with powdery and crushed stone-like calcifications with or without associated tumor mass on the mammogram, had favorable survival rates irrespective of basal phenotype or histologic tumor distribution. Circular-appearing tumors with a nonbasal phenotype and unifocal status showed lower risk of breast cancer death (2.9% for small tumors; 6.3% for large tumors) than those with nonbasal phenotype and multifocal appearance (23.0% 10-year risk). For unifocal tumors with basal phenotype, patient survival was strongly dependent on the tumor size: 10-year risk of death was 2.9% for a tumor < 15 mm, and 30.4% for a tumor ≥ 15 mm.

Mammographic tumor appearance usually is the first available breast cancer prognostic indicator, and these data suggest that mammographic tumor appearance combined with other indicators would be useful for treatment planning. Breast tumors with powdery and crushed stone-like mammographic tumor appearance (see Figure 3) may indicate a lower risk, and treatment and therapy provided for these cases may not need to be aggressive. In contrast, tumors with casting-type calcifications or architectural distortion, regardless of size, are very aggressive breast tumors. The potential for early detection of these tumors by mammography may be limited, and at the time of diagnosis both types require the use of magnetic resonance imaging (MRI) and ultrasonography for determining the full extent of breast tissue involved. These tumors also will benefit from large-section histology assessment, extensive surgery, and regular, intensive follow-up. Although these tumors represent a small proportion of cases, they are disproportionately responsible for a larger proportion of breast cancer deaths [5]. In this series, casting type tumors accounted for 6% of the invasive breast cancers, but 12% of the breast cancer deaths.

Given these observations, it may be unnecessary to test for the basal phenotype in patients with powdery, stellate, or crushed stone-like appearance tumors because it is so rare. However, testing for the basal phenotype is indicated for circular-type tumors, in addition to tumors with a circular/oval-shaped appearance on the mammogram, as shown previously by Luck et al. who also demonstrated that screen-detected breast tumors with the basal phenotype were more likely to reveal mammographic tumor appearance features of an ill-defined mass compared with breast tumors having the nonbasal phenotype [30].

The basal phenotype elevated the risk for breast cancer death 2.68-fold, even after adjusting for mammographic tumor appearance and tumor attributes. However, for breast cancer detected at a very early stage when tumors measure < 15 mm, prognosis is favorable, even for the basal phenotype. The difference in survival between basal and nonbasal phenotype tumors is observed in larger tumors, as the basal phenotype is more predictive of breast cancer death in tumors ≥ 15mm. This not only emphasizes the need for strict adherence to screening intervals to detect the more aggressive basal phenotype tumor at an early stage but also affects the choice of treatment modalities. Referring to Figure 3, among the basal phenotype unifocal circular lesions < 15 mm, the 10-year risk of death is 2.9% compared with 30.4% for the lesions ≥ 15 mm. Given the fact that conventional approaches to

Figure 3: Combining mammographic appearance, histologic tumor distribution, and molecular subtype where relevant to classify cases into risk groups and 10-year breast cancer death. BCS, breast conserving surgery; Mast, mastectomy; Adj Tx, adjuvant therapy; R, radiotherapy; C, chemotherapy; H, Hormone therapy.

treatment planning lead to similar therapy for women with a unifocal tumor with the basal phenotype regardless of tumor size, these data suggest that the need for aggressive treatment should be reconsidered if the tumor size is small.

Determining histological tumor distribution is important for tumors of all mammographic tumor appearance types, particularly for stellate type and circular nonbasal types as they account for 46% and 24% of the breast cancers. Figure 3 shows that circular, unifocal, and nonbasal tumors are a low risk group regardless of tumor size and thus these patients also may not need aggressive treatment. Further, the risk of breast cancer death for stellate type breast cancer is highly dependent on histologic tumor distribution. The one-third of these tumors that are unifocal and size <15mm may not need aggressive treatment, whereas the one-third with larger size or multifocality needs complete surgical removal, and thus use of MRI to specify the extent of disease should be considered to insure optimal surgical treatment and adjuvant therapies as appropriate.

There are two main concerns over the methodology. First, the validation design used here is with external validation that is more reliable and stringent than the conventional cross-validation (the test cohort with part of the samples and the validated cohort with the rest of samples) as the external cohort is independent of the test cohort. However, the main limitation is that the follow-up time of the validation cohort is too short to have sufficient numbers of breast cancer death. However, similar findings were noted on the comparison of the test cohort and the validated cohort in the univariate analysis (Table S1-S8), and also the comparison of the expected and the observed survival curves in different time periods by risk groups (Figure S1) also was similar. A larger external validation cohort is therefore required to verify the proposed new classification. Moreover, the effect of treatment and therapy should not be taken as one part of the attribute-based predictors, but rather decision factors determined by surgeons and oncologists (see the last two column of Figure 3) and could vary with time periods due

to the advance in treatment. This period effect should be taken into account into the model, when different periods are considered, for predicting the prognosis but not for risk classification.

The second is that other causes of death are considered as independent censorship rather than informative censorship. To relieve this concern, we analyzed the data with the competing risk model. The results are very similar between the competing risk model and the conventional survival model that treated other causes of death as censored cases (Table S9). The only difference is that the use of the competing risk model may affect the statistical power of some variables such as the basal phenotype.

There is the third limitation related to the problem of collinearity between basal phenotype and histologic tumor distribution when they were assessed as independent prognostic predictors. Due to limited sample size, we needed to combine three tumor categories (the stellate, powdery calcifications, and crushed stone-like calcification) as one group. In the future, it will be preferable to analyze these three categories separately in a larger study.

5. Conclusions

The combined information regarding mammographic tumor features, histologic tumor distribution and basal phenotype enables us to distinguish groups with different degrees of risk of breast cancer death. The model sequentially incorporates different dimensional attributes according to the chronological order in which the information is obtained. The combined imaging biomarker information for risk classification may be relevant for determining the need for molecular testing or other investigations, planning treatment, and determining the most appropriate clinical surveillance schedule for breast cancer patients. Our prognostic model was validated on an independent dataset. However, we believe further validation or refinement using larger tumor series is required and should be pursued, particularly, to elucidate intercorrelational but complementary role of imaging biomarkers.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Additional Points

This paper was done by The Swedish Two County Trial Research Group. The members of The Swedish Two County Trial Research Group are presented in the authors' list in the front matter of the paper.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary materials contain relationships between mammographic appearance and biomarkers (Tables S1-S6), the estimated results of Cox regression model in both test and validation cohorts (Tables S7), the estimated results of Cox regression model with and without consideration of competing risk of death (Table S9), and model validation with observed and expected numbers by risk groups (Table S8 and Figure S1). The table and figure legends are as follows. Table S1: relationship of mammographic appearance to basal phenotype, histologic tumor distribution, and conventional tumor attributes in test cohort. Table S2: relationship between basal phenotype and mammographic appearance, histologic tumor distribution, and tumor attributes in the test cohort. Table S3: relationship between histologic tumor distribution and mammographic appearance, basal phenotype, and tumor attributes in the test cohort. Table S4: relationship of mammographic appearance to basal phenotype, histologic tumor distribution, and conventional tumor attributes in validation cohort. Table S5: relationship between basal phenotype and mammographic appearance, histologic tumor distribution, and tumor attributes in the validation cohort. Table S6: relationship between histologic tumor distribution and mammographic appearance, basal phenotype, and tumor attributes in the validation cohort. Table S7: unadjusted (crude HR) and adjusted hazard ratios (aHR) for the effects of mammographic appearance, basal phenotype, histologic tumor distribution, and conventional tumor attributes on the risk for breast cancer death by test and validation cohort. Table S8: the observed and expected number of breast cancer death (BCD) by four risk groups. Table S9: results of univariate and multivariable Cox regression models before and after considering the competing risk of death in the test cohort. Figure S1: the observed (O) and expected (E) survival curve by four risk groups. (a) Low risk, (b) intermediate risk, (c) high risk, and (d) extremely high risk. *(Supplementary Materials)*

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