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# Treatment Strategies and Survival Outcomes in Breast Cancer

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Edited by

**Kwok-Leung Cheung**

Printed Edition of the Special Issue Published in *Cancers*

# **Treatment Strategies and Survival Outcomes in Breast Cancer**



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Special Issue Editor

**Kwok-Leung Cheung**

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## About the Special Issue Editor

**Kwok-Leung Cheung** is Professor of Breast Surgery and Medical Education, University of Nottingham and Honorary Consultant Breast Surgeon, University Hospitals of Derby and Burton. He qualified and trained at Queen Mary Hospital, Hong Kong. Upon completion of general surgery training, he conducted breast cancer research at the University of Nottingham, with a DM awarded in 2001.

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Editorial

# Treatment Strategies and Survival Outcomes in Breast Cancer

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Treatment strategies for breast cancer are wide-ranging and often based on a multi-modality approach, depending on the stage and biology of the tumour and the acceptance and tolerance of the patient. They may include surgery, radiotherapy, and systemic therapy (endocrine therapy, chemotherapy, and targeted therapy). Advances in technologies such as oncoplastic surgery, radiation planning and delivery, and genomics, and the development of novel systemic therapy agents alongside their evaluation in ongoing clinical trials continue to strive for improvements in outcomes. In this Special Issue entitled, ‘Treatment strategies and survival outcomes in breast cancer’, a number of original research articles are included covering a diversity of studies, from pre-clinical and translational biomarker studies to clinical trials and population-based studies. They evaluated survival and other outcomes, including quality of life, in the context of pre-diagnosis (screening), as well as early and advanced stages of breast cancer.

With the established survival benefits of prophylactic mastectomy in women with BRCA genetic mutations, the procedure is increasingly being performed on the contralateral breast following diagnosis of breast cancer. Teoh et al. conducted a review of the literature which mostly consisted of retrospective studies with less than optimal data quality [1]. The evidence suggests a reduction of incidence of contralateral breast cancer following the procedure in those with ‘high risk’, notably those with BRCA genetic mutations, whereas survival benefits are uncertain. The overall benefits in other risk categories are even more doubtful. In the area of pre-invasive cancer, Sieuwerts et al. observed in cases of ductal carcinoma in situ an upregulation of APOBEC3B, which was previously seen in invasive carcinoma and known to be associated with poor prognosis, suggesting its potential role in early carcinogenesis [2]. There are two studies on screening. Heller looked at approximately 993,000 individuals using a national database, aiming to see why screening did not appear to decrease the incidence of stage IV breast cancer [3]. They found that among those diagnosed up front with stage IV disease, 37.6% had aggressive tumours as compared to 5.1% in those with stage 1 disease, suggesting that the two groups are from different populations with different tumour phenotypes. Regarding screening, over-diagnosis has been coined as the main concern. Fann et al. evaluated the 15-year adjusted cumulative survival of breast cancer in a cohort in Sweden, and noted that the majority of survivors could be attributed to cure arising from screening and subsequent treatments [4]. According to their interpretation, over-diagnosis had minimal contribution.

For primary breast cancer, Corradini et al. analysed the oncological outcomes of 7565 cases of breast cancer in a case-controlled cohort study comparing breast conserving surgery followed by radiotherapy with mastectomy, showing that the former was associated with better recurrence control and survival, and as such recommended physicians to encourage women to receive such treatment [5]. While the findings are interesting, provocative and continue to be reassuring in terms of the efficacy of breast conserving surgery, their applications must be cautioned. The findings have not been consistently shown by randomised controlled trials and must be further investigated before a change in practice is implemented. This Special Issue also contains a few studies related to breast

cancer in older patients. In a population-based registry study in the Netherlands, survival in these patients was found to be poorer when compared to their younger counterparts, and the observation was shown to be associated with a proportionately reduced use of surgery and increased use of primary endocrine therapy [6]. As discussed by the researchers, this phenomenon has been picked up in other studies and changes in treatment guidelines have since been made. While surgery is now the primary treatment of choice in this population as in the younger one, alternative treatments such as primary endocrine therapy may still be appropriate, especially in patients with competing causes of death due to significant comorbidities. Given this, and other needs to appropriately select treatments, including primary and adjuvant therapies in this challenging population, biomarker studies play a very important role in translational research. Three such biomarkers—LKB1 [7] and cytoplasmic cyclin E [8] (poor prognostic in the older (>70 years) population), and HDAC5 [9] (poor prognostic in the very young (<35 years) patients)—have been found to be associated with age. Furthermore, other conventional and emerging prognostic and predictive factors were investigated and reported in this Special Issue. Kim et al. highlighted the use of high lymph-node ratio following axillary surgery as an indicator of poor prognosis and the need for radiotherapy to the supraclavicular fossa in a retrospective study [10]. However, sentinel node biopsy has now become the standard axillary staging procedure, making the precise calculation of the ratio difficult. As a result, its potential clinical application is likely to be limited. In addition, Abdel-Fatah studied an emerging biomarker, ERCC1, a DNA excision repair protein, and noted its potential prognostic significance and ability to predict response in neoadjuvant chemotherapy [11]. In a different study using a cell line model, Gaule et al. identified the potential role of combining dasatinib and a c-Met inhibitor, in order to combat dasatinib resistance in triple negative breast cancer [12].

In the context of metastatic breast cancer, the Special Issue contains two pieces of work focusing on important new targeted therapies currently licensed for clinical use, CDK4/6 inhibitors and anti-HER2 therapies. Rossi et al. carried out a network meta-analysis comparing the combination use of individual CDK4/6 inhibitors with fulvestrant or an aromatase inhibitor [13]. They found that CDK4/6 inhibitors have similar efficacy when combined with an aromatase inhibitor in the first-line treatment of hormone receptor positive disease, and are superior to either endocrine agent as monotherapy, regardless of any other patient or tumour characteristics. While this may be seen as reassuring for those who are strong supporters of using such a combination despite the concerns on increased toxicity, the authors admitted the limitations of their meta-analysis, including not using actual patient data, the lack of uniformity in terms of prior use of endocrine therapy, and the fact that some trials employed non-standard fulvestrant dose (250 mg, rather than 500 mg). On the other hand, the PRAEGNANT Real-World Breast Cancer Registry study reviewed the landscape of using anti-HER2 therapies [14]. Both novel therapies (pertuzumab/trastuzumab and T-DM1) are utilised in a high proportion of HER2 positive breast cancer patients. Most patients were found to receive T-DM1 after pertuzumab/trastuzumab in a real-world setting. The Special Issue contains two other interesting studies regarding this disease stage. Keup et al. advocated a ‘comprehensive’ liquid biopsy, including both cell-free DNA mutational and circulating tumour cell transcriptional analyses, which could increase the chance of identifying actionable targets at which to direct therapeutic strategies [15]. Pelizzari identified the change in plasma LDH levels as a potential cost-effective biomarker of prognosis in the early course of systemic therapy [16]. According to the results of their study, patients who maintained elevated LDH levels after 12 weeks of first-line treatment experienced worse survival outcomes when compared to those with stable normal LDH levels, even after adjustment for other prognostic factors.

Finally, as opposed to survival outcomes, Hong et al. carried out a systematic review and meta-analysis of randomised controlled trials to investigate quality of life as another important treatment outcome for breast cancer [17]. Their work showed that exercise interventions improved quality of life and that the ‘time of session’ (longer than 45 minutes) appeared to be crucial in achieving significant improvement.

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Review

# Contralateral Prophylactic Mastectomy in Women with Unilateral Breast Cancer Who Are Genetic Carriers, Have a Strong Family History or Are just Young at Presentation

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**Abstract:** The uptake of contralateral prophylactic mastectomy is rising with increasing trends that are possibly highest in the USA. Whilst its role is generally accepted in carriers of recognized high-risk predisposition genes such as *BRCA1* and *BRCA2* when the affected individual is premenopausal, controversy surrounds the benefit in less understood risk-profile clinical scenarios. This comprehensive review explores the current evidence underpinning the role of contralateral prophylactic mastectomy and its impact on contralateral breast cancer risk and survival in three distinct at-risk groups affected by unilateral breast cancer: known genetic carriers, those with strong familial risk but no demonstrable genetic mutation and women who are of young age at presentation. The review supports the role of contralateral prophylactic mastectomy in “high risk” groups where the evidence suggests a reduction in contralateral breast cancer risk. However, this benefit is less evident in women who are just young at presentation or those who have strong family history but no demonstrable genetic mutation. A multidisciplinary and personalized approach to support individuals in a shared-decision making process is recommended.

**Keywords:** contralateral prophylactic mastectomy; contralateral breast cancer; BRCA; CHEK2; PALB2; ATM; mutation carriers; family history; survival

## 1. Introduction

The incidence of women with breast cancer who elect to undergo contralateral prophylactic mastectomy is steadily increasing, with preponderance amongst Caucasians, young women, and those with a higher socioeconomic status [1,2]. A study of 496,488 women with unilateral Stage I–III breast cancer, from the Surveillance, Epidemiology, End Results (SEER) Program database demonstrated an increase in contralateral prophylactic mastectomy rates performed for unilateral invasive breast cancer from 3.9% in 2002 to 12.7% in 2012 [3]. This effect was reproduced in a National Cancer Database review of 553,593 patients, showing an increase in contralateral prophylactic mastectomies from 4.1% in 2003 to 9.7% in 2010. This finding was most marked in young women, where those <45 years ( $n = 73,888$ ) showed an increase from 9.3% in 2003 to 26.4% in 2010 [4].

Factors that contribute to this decision include patient age, disease stage, previous breast biopsies, genetic predisposition or family history of breast cancer, fear of recurrence, concern with cosmetic symmetry and physician recommendation [1,5–8].

Patients tend to overestimate their risk of developing a contralateral breast cancer [9,10] as well as the extent of risk reduction conferred by contralateral prophylactic mastectomy [9,11]. Interestingly,



whilst 43.9% of women with breast cancer considered contralateral prophylactic mastectomy, only 38.1% were aware that it did not improve survival, highlighting the importance of patient education [12].

Improvements in modern multidisciplinary management have led to a reduction in the incidence of contralateral breast cancer from approximately 0.6% to 0.2–0.5%/year [13]. Consequently, the role of contralateral prophylactic mastectomy and the context in which it is supported is debatable.

This comprehensive review explores the current evidence underpinning the role of contralateral prophylactic mastectomy and its impact on contralateral breast cancer risk and survival in three high-risk groups affected by breast cancer: (i) genetic carriers, (ii) strong family history with no demonstrable mutation, and (iii) young women.

## 2. Methods

A comprehensive literature review was performed, assessing all studies published in the English literature from 1974 to March 2019 across Embase and Medline search engines. Search terms “contralateral prophylactic mastectomy”, “unilateral breast cancer”, “BRCA”, “TP53”, “PALB2”, “CHEK2”, “ATM”, “mutation carrier”, “family history”, “young women”, “non-genetic carriers”, “overall survival”, “disease-free survival”, “contralateral breast cancer” and “risk” were included. Relevant references from identified papers were also included.

## 3. BRCA 1/2 Carriers with Breast Cancer

### 3.1. BRCA 1/2 Carriers and Contralateral Breast Cancer Risk

BRCA carriers with breast cancer carry a higher risk of contralateral breast cancer, 23.7% (95% CI 17.6–30.5), compared with non-carriers, 6.8% (95% CI 4.2–10), respectively, (RR 3.56, 95% CI 2.50–5.08;  $p < 0.001$ ). This risk was higher in BRCA1 compared to BRCA2 carriers (RR 1.42, 95% CI 1.01–1.99;  $p = 0.04$ ) [14]. In a Dutch multicentre study of 6294 invasive breast cancer patients  $\leq 50$  years, the risk of contralateral breast cancer for BRCA1/2 carriers at a median follow-up of 12.5 years was shown to be 2–3 times higher compared to non-carriers (HR 3.31, 95% CI 2.41–4.55;  $p < 0.001$  and 2.17, 95% CI 1.22–3.85;  $p = 0.01$  respectively). The 10-year cumulative contralateral breast cancer risk following the initial breast cancer diagnosis was 21.1% for BRCA1, 10.8% for BRCA2 and 5.1% for non-carriers [15]. These findings were confirmed in a recent multicentre study where the 10-year cumulative risk was 25.1% (95% CI 19.6–31.9) for BRCA1, 13.5% (95% CI 9.2–19.1) for BRCA2 and 3.6% (95% CI 2.2–5.7) for non-carriers [16].

The age of first breast cancer diagnosis is a significant predictor of contralateral breast cancer risk in BRCA carriers [17–19]. Risk estimates vary in the literature, ranging from 23.7–30.7% in young women (<40 years) across BRCA1/2 carriers combined (BRCA1: 24–32%; BRCA2: 17–29%) [19–24]. This risk is lower in the >40 years age group, ranging from 8.4–21% (BRCA1: 11–52%; BRCA2: 7–18%) [15,17,19–22,24,25]. Similar results were shown in another study demonstrating a 10-year cumulative contralateral breast cancer risk of 23.9% (BRCA1: 25.5%; BRCA2: 17.2%) in patients <41 years, compared to 12.6% in the 41–49 year group (BRCA1 15.6%; BRCA2 7.2%) [15].

In a retrospective study of 1042 BRCA1/2 carriers with breast cancer, Graeser demonstrated that the 25-year cumulative contralateral breast cancer risk for BRCA1 carriers with the first breast cancer diagnosis at age <40 years, 40–50 years and >50 years, was 62.9% (95% CI 50.4–75.4), 43.7% (95% CI 24.9–62.5) and 19.6% (95% CI 5.3–33.9) respectively. In BRCA2 carriers, the corresponding rates were 63% (95% CI 32.8–93.2), 48.8% (95% CI 22.7–74.9) and 16.7% (95% CI 1.0–32.4) for the respective age groups [19].

### 3.2. Contralateral Prophylactic Mastectomy and Risk of Contralateral Breast Cancer

Contralateral prophylactic mastectomy reduces the risk of contralateral breast cancer in BRCA mutation carriers [14,26,27]. This risk reduction has been reported to be in the range of 91% [27]. This

is further supported by a meta-analysis showing that contralateral prophylactic mastectomy resulted in a 93% reduction in contralateral breast cancer risk (RR 0.072; 95%CI 0.035–0.588) [26].

### 3.3. Contralateral Prophylactic Mastectomy and Survival

There is conflicting evidence on whether contralateral prophylactic mastectomy improves survival in *BRCA* carriers with breast cancer [14,26–31] (Table 1). In a multicenter, retrospective study of 242 *BRCA* carriers with breast cancer, contralateral prophylactic mastectomy was associated with improved overall survival on multivariate analysis, having adjusted for risk-reducing salpingo-oophorectomy (HR 0.49, 95% CI 0.29–0.82) [30]. Similar findings have been reported in other cohort studies [27,29–31].

**Table 1.** Studies looking at the impact of CPM on CBC risk and survival in *BRCA1/2* mutation carriers.

Author	Year	Study Type	Follow up	Patient	Findings
Li [26]	2016	Meta-analysis (2 RC/1 PC/1 RCC)	n/a	4/4574 studies (1672 individuals)	CPM significantly decreased CBC risk in <i>BRCA1/2</i> mutation carriers (RR 0.072; 95% CI, 0.035–0.148). CPM is associated with a decrease in “all-cause” mortality (HR 0.512; 95% CI 0.368–0.588)
Valachis [14]	2014	Meta-analysis (1 RCC/1 RC)	n/a	2/13 studies	CPM was not associated with a benefit in BCSS HR 0.78 (95% CI 0.44–1.39, $p = 0.40$ )
Copson [32]	2018	Prospective cohort	Median 8.2 years	21 <i>BRCA</i> carriers/10 non-carriers, with TNBC	CPM conferred no difference in 5-year OS between <i>BRCA</i> carriers and non-carriers with TNBC 83% (95% CI 74–89) vs. 74% (95% CI 69–78) HR 0.98 (95% CI 0.58–1.65), $p = 0.94$
Heemskerck-Gerritsen [30]	2015	Multicentre retrospective cohort	Median 11.4 years	242/583(52%) carriers with BC who underwent CPM	CPM improved OS HR 0.49 (0.29–0.82)
Metcalf [29]	2014	Retrospective observational	Median follow up 14.3 yrs (0.1–20.0)	390 <i>BRCA1/BRCA2</i> carriers with a positive family history	At 20 years follow up, CPM was associated with a 48% reduction in death from breast cancer (HR 0.52; $p = 0.03$ ). * Not significant on propensity score adjusted analysis
Evans [31]	2013	Retrospective case-control	Median 9.7 years	105/698 (15%) <i>BRCA 1/2</i> carriers with BC who underwent CPM	CPM improves OS 89% (CPM) vs. 71% (non-CPM) at 10 year follow up ( $p < 0.001$ )
Van Sprundel [27]	2005	Retrospective cohort	Mean 3.5 years	69/148 (47%) <i>BRCA 1/2</i> carriers with BC who underwent CPM	CPM reduced the risk of CBC in <i>BRCA1/2</i> carriers by 91% No significant difference in OS between CPM and non-CPM group HR 0.35, $p = 0.14$ (adjusted for prophylactic oophorectomy)
Brekelmans [28]	2007	Retrospective case-control	Median 4.3 years	260 <i>BRCA 1/2</i> carriers with BC vs. 759 non-carriers	CPM conferred no difference in BCSS HR 0.98 (95% CI 0.5–0.91, $p = 0.96$ )

RC: retrospective cohort; RCC: Retrospective case-control; PC: prospective cohort; BCSS: breast-cancer-specific; OS overall survival; CPM: contralateral prophylactic mastectomy; BC: breast cancer; CBC: contralateral breast cancer; TNBC: triple negative breast cancer; HR: hazard ratio.

In a retrospective study by Van Sprundel, contralateral prophylactic mastectomy was associated with superior overall survival compared to active surveillance at 5-year follow up (94% vs 77%,  $p = 0.03$ ). However, this difference was not significant once adjusted for prophylactic oophorectomy (HR 0.35,  $p = 0.14$ ) [27]. Notably, Metcalfe observed a survival benefit only in the second decade of follow-up following initial breast cancer diagnosis (HR 0.52, 95% CI 0.29–0.93) but not during the first 10 years of follow-up (HR 0.65, 95% CI 0.34–1.22) [29].

A meta-analysis by Valachis demonstrated no difference in breast-cancer-specific survival between *BRCA* carriers who underwent contralateral prophylactic mastectomy against those who did not (HR

0.78, 95% CI 0.44–1.39;  $p = 0.40$ ) [14]. However, a meta-analysis including two additional studies demonstrated a decrease in “all-cause” mortality [26]. To further add to the ambiguity, a recent prospective study showed that contralateral prophylactic mastectomy conferred no benefit in 5-year overall survival between *BRCA* carriers and non-carriers with triple negative breast cancer [32]. The available findings should be interpreted with caution as they are mostly based on retrospective studies that may contain recognized and unrecognized biases.

#### 4. “Other” Genetic Carriers (*CHEK2*, *TP53*, *ATM*, *PALB2*, *PTEN*, *CDH1*) with Breast Cancer

Mutations in *CHEK2*, *TP53*, *ATM*, *PALB2*, *PTEN*, and *CDH1* account for a small fraction of familial breast cancers. The available studies are sparse and primarily family-based, with potential ascertainment bias. It should be noted that the existing literature focusses mainly on relative rather than absolute risk estimates.

##### 4.1. “Other” Genetic Carriers and Contralateral Breast Cancer Risk

###### 4.1.1. *CHEK2* Mutation Carriers and Contralateral Breast Cancer Risk

In a recent meta-analysis, Akdeniz demonstrated an increased contralateral breast cancer risk for *CHEK2*\* 1100delC carriers (RR 2.75, 95% CI 1.77–4.27) [33]. This mutation is associated with bilateral disease and an increased risk of bilateral breast cancer which varies between two to six-fold [15,34–40] (Table 2). It has been suggested that *CHEK2* carriers may be more sensitive to ionizing radiation that may contribute to contralateral breast cancer rates in patients receiving adjuvant radiotherapy following breast conserving surgery [40,41]. The true impact of radiation in this context is questionable as consistently increased contralateral breast cancer risk has been demonstrated in patients treated with or without radiotherapy (HR 4.12, 95% CI 2.49–6.83 and HR 3.17, 95% CI 1.36–7.35, respectively) [39,41].

###### 4.1.2. *TP53* Mutation Carriers and Contralateral Breast Cancer Risk

There are no studies estimating contralateral breast cancer risk in *TP53* carriers with breast cancer.

###### 4.1.3. *ATM* Mutation Carriers and Contralateral Breast Cancer Risk

In a multicentre, population-based, case-control study, Concannon suggested that four common variants of *ATM* (*c.1899-55T>G*; *c.3161C>G*; *c.6348-54T>C* and *c.5558A>T*) were associated with a lower contralateral breast cancer risk (overall RR 0.8, 95% CI 0.6–0.9) compared to those with rare, missense *ATM* mutations. The protective mechanisms may occur through an alteration in *ATM* activity as an initiator of DNA damage response or through its role in *TP53* regulation [42]. Bernstein suggested that common *ATM* variants may exert a protective effect and reduce contralateral breast cancer risk, while rare *ATM* missense, deleterious variants may act synergistically with radiation exposure to increase this risk [43]. In this study, the variants: *c.1899-55T>G* (RR 0.5, 95% CI 0.3–0.8), *c.3161C>G* (RR 0.5, 95% CI 0.3–0.9), *c.5558A>T* (RR 0.2, 95% CI 0.1–0.6), and *c.6348-54T>C* (RR 0.2, 95% CI 0.1–0.8) were associated with significantly reduced risk. On the other hand, female carriers of any rare missense *ATM* variant, who received radiation therapy for their first breast cancer, had a significantly elevated contralateral breast cancer risk compared to unexposed women (RR = 2.8 for <1.0 Gy dose and RR = 3.3 for ≥1.0 Gy dose to the contralateral breast).

The direct relationship between the presence of *ATM* variants and the overall risk of contralateral breast cancer remains controversial, although the combination of radiotherapy and certain *ATM* missense variants appears to accelerate tumour development [44].

**Table 2.** Studies looking at CBC risk and survival in *CHEK2\* 1100delC* mutation carriers.

Author	Year	Study	Median Follow up	N	Findings
Akdeniz [33]	2019	Meta-analysis	N/A	68 studies	CBC risk by mutation carriers <i>BRCA1</i> RR 3.7 (95% CI, 2.8–4.9) <i>BRCA2</i> RR 2.8 (95% CI, 1.8–4.3) <i>CHEK2* 1100delC</i> RR 2.7 (95% CI, 2.0–3.7)
Kriege [39]	2014	Retrospective, multicentre cohort study	6.8 years	193/4722 (4.1%) BC patients with <i>CHEK2* 1100delC</i> mutation	Higher risk of CBC HR 3.97 (95% CI 2.59–6.07) 10-year risk of CBC is 28.9%
Weischer [37]	2012	Meta-analysis	6 years	459/25571 (1.8%) BC patients with <i>CHEK2* 1100delC</i> mutation	20-year cumulative risk of developing BC is 25–30% (HR 3.52) No comment on CBC rates
Mellemkjaer [40]	2008	Population based, multicentre cohort study	N/A	17/2103 (0.8%) BC patients with <i>CHEK2* 1100delC</i>	No significant association between <i>CHEK2* 1100delC</i> mutation and CBC
Broeks [41]	2004	Case study	N/A	15/233 (6.4%) <i>CHEK2* 1100delC</i> mutation carriers with BBC 2/191 (1%) <i>CHEK2* 1100delC</i> mutation carriers with UBC	Increased risk of CBC in carriers OR 6.5 (95% CI 1.5–28.8, $p = 0.005$ )
Schmidt [36]	2007	Retrospective cohort study	Median 10.1 years	54/1479 (3.7%) pre-menopausal BC patients with <i>CHEK2* 1100delC</i> mutation	<i>CHEK2* 1100delC</i> mutation carriers: Increased risk of ipsilateral second breast cancer HR 2.1 (95% CI 1.0–4.3; $p = 0.049$ ) Increased risk of CBC HR 1.7 (95% CI 1.2–2.4) Worse breast-cancer-specific survival HR 1.4 (95% CI 1.0–2.1; $p = 0.072$ ) Worse recurrence-free survival HR 1.7 (95% CI 1.2–2.4; $p = 0.06$ )
De Bock [45]	2004	Prospective cohort	Median 3.8 years	34 BC patients with <i>CHEK2* 1100delC</i> mutation; 102 BC patients with no mutation	Compared to non-carriers, <i>CHEK2* 1100delC</i> mutation carriers: Increased risk of CBC compared RR = 5.74 (95% CI 1.67–19.65) Increased risk of distant metastasis RR 2.81 (95% CI 1.2–6.58) Worse DFS RR = 3.86 (1.91–7.78) No difference in overall survival. Mutation carriers more frequently had a 1st or 2nd degree female relative with breast cancer ( $p = 0.03$ )

BC: breast cancer; CBC: contralateral breast cancer; BBC: bilateral breast cancer; UBC: unilateral breast cancer; RR: relative risk; DFS: disease-free survival; ER: oestrogen receptor; HR: hazard ratio.

#### 4.1.4. *PALB2* Mutation Carriers and Contralateral Breast Cancer Risk

There are no studies estimating contralateral breast cancer risk in *PALB2* carriers with breast cancer.

#### 4.1.5. *CDH1* Mutation Carriers and Contralateral Breast Cancer Risk

There are no studies on the risk of contralateral breast cancer in *CDH1* carriers.

#### 4.1.6. *PTEN* Mutation Carriers and Contralateral Breast Cancer Risk

There are no studies estimating contralateral breast cancer risk in *PTEN* carriers with breast cancer.

#### 4.2. Contralateral Prophylactic Mastectomy and Risk of Contralateral Breast Cancer

No studies have investigated the role of contralateral prophylactic mastectomy in the risk reduction of contralateral breast cancer in patients with a diagnosis of breast cancer that harbor a genetic mutation in non-*BRCA1/2* genes (*CHEK2*, *TP53*, *ATM*, *PALB2*, *CDH1* and *PTEN*).

#### 4.3. Contralateral Prophylactic Mastectomy and Survival

There is no data to support any survival benefit from contralateral prophylactic mastectomy in this group of patients (“other” genetic carriers). This would be even more challenging in those with *TP53*, *CDH1* and *PTEN* mutations because of the additional competing cancer risk. In view of the limited evidence, no further comment can be made, except to reinforce that contralateral prophylactic mastectomy should be considered on an individual basis for women with unilateral breast cancer in this group.

### 5. Familial Breast Cancers with no Demonstrable Genetic Mutations

#### 5.1. Familial Breast Cancers with No Demonstrable Genetic Mutation and Contralateral Breast Cancer Risk

A positive family history remains a strong risk factor for contralateral breast cancer, even after excluding mutation carriers [46–48]. Table 3 summarizes the current literature on the impact of positive family history on contralateral breast cancer risk and survival. In a multicentre, population-based, case-control study of 1521 contralateral breast cancer cases against 2212 matched controls of unilateral breast cancer, Reiner demonstrated that non-mutation carriers with any 1st or 2nd degree relative of breast cancer had a nearly two-fold increased contralateral breast cancer risk (RR 1.8, 95% CI 1.3–2.4), compared to individuals without a family history. This risk is similar to that shown in previous studies [49,50]. In this non-mutation carrier group, a 1st degree family history of bilateral breast cancer increased the contralateral breast cancer risk by more than three-fold (RR 3.4, 95% CI 1.5–7.4). Where there is only an affected 2nd degree relative, the individual is at a 40% increased risk compared to an individual without a family history. The 10-year absolute contralateral breast cancer risk in non-mutation carriers with a 1st or 2nd degree family history is 8.3% (95% CI 5.5–12.6) and 6.6% (95% CI 4.4–10) respectively [46].

**Table 3.** Studies looking at the impact of positive FH of breast cancer on CBC rates, disease-free and overall survival.

Author	Year	Study Type	Follow up	Patients	Findings
Reiner [46]	2018	Multicentre, population-based, case-control study	Not stated	1521 CBC cases with 2212 UBC controls	A 1st degree relative with BC confers increased risk of CBC RR 1.9 (95% CI 1.6–2.3)
					A 1st degree relative with BBC confers the highest risk of CBC RR 3.4 (95% CI 2.4–5)
Kuchenbaecker [20]	2017	Prospective, multicentre, cohort study	Median 4 years (2–7)	3886 eligible for breast cancer analysis <i>BRCA1</i> ( <i>n</i> = 2276); <i>BRCA2</i> ( <i>n</i> = 1610)	A 2nd degree relative increases the risk of CBC RR 1.4 (95% CI 1.2–1.7)
					Any 1st degree relative with breast cancer confers a 10-year AR of developing CBC of 8.1% (95% CI 6.7–9.8). The 10-year AR increases to 13.5% (95% CI 8.8–20.8) if this relative was <40 years at age of diagnosis. The 10-year AR is highest at 36% (95% CI 14.5–50.5) if the first degree relative was diagnosed with BBC at age <40 years. On subgroup analysis and exclusion of mutation carriers i.e., <i>BRCA</i> , <i>ATM</i> , <i>PALB2</i> and <i>CHEK2</i> , the increased 10-year AR associated with a 1st degree relative and a 1st degree relative with BBC remained significant similar to above-reported.
Bernstein [51]	1992	Prospective cohort study	Mean 52 months	136/4550 (2.9%) patients with CBC and varying familial risk	Increased risk if ≥two 1st or 2nd degree relatives with breast cancer compared to no family history of BC; HR 1.99 Did not evaluate the effect of FH on CBC risk
					Increased risk of CBC ~2x with a 1st degree relative with BC Increased risk of CBC ~3x if 1st degree relative was diagnosed at a young age (<35 years)
Ji [52]	2007	Population based, national database study	Not stated	56190 invasive and 6841 in situ BC patients	The risk of metachronous CBC measured by SIRs was higher with primary in situ disease compared to invasive cancer. SIR for metachronous CBC in women diagnosed with invasive BC: <45 years: 5.12 (95% CI 4.47–5.85) 45–55 years: 1.95 (95% CI 1.76–2.16) >55 years: 1.49 (95% CI 1.37–1.61)
					SIR for metachronous CBC in women diagnosed with 1st invasive BC and have: A positive FH 2.74 (95% CI 2.3–3.23) No FH 1.85 (95% CI 1.75–1.96) SIR for metachronous CBC in women diagnosed with in situ disease: <45 years: 5.12 (95% CI 4.47–5.85) 45–55 years: 1.95 (95% CI 1.76–2.16) >55 years: 1.49 (95% CI 1.37–1.61)

Table 3. *Cont.*

The Impact of Positive FH of Breast Cancer on CBC Rates, Disease-Free and Overall Survival					
Author	Year	Study Type	Follow up	Patients	Findings
Narod [53]	2016	Population based, national database study	Not stated	4839 CBC patients out of 84819 patients with BC * (5.7%)	<p>Young age at 1st BC diagnosis and a maternal cancer history increases the risk of CBC</p> <p>The 15-year cumulative risk of CBC was:                      8.8% (95% CI 8.5–9.1) in the general population (regardless of maternal BC status)                      12% (95% CI 11–13) in maternal UBC                      13% (95% CI 9.5–17) in maternal BBC</p> <p>A maternal cancer history of UBC at an early age conferred the daughter a lifetime CBC risk of 35% (95% CI 25–46)                      * Mutation carriers not excluded as information not available from cancer registry</p>
Vaittinen [54]	2000	Population based, national database study	Not stated	2529/72,092 (3.5%) CBC patients. 147 (5.8%) of CBC cases with 1st degree relative	Modest elevation in CBC risk for women with an affected 1st degree relative RR of 1.53
Boughey [55]	2010	Retrospective cohort	Median 17.3 years	385 patients with a positive FH; 385 matched controls	<p>Patients with stage I or II BC and a positive family history who underwent CPM had:                      A 95% reduction in CBC rates; adjusted HR 0.05 (95% CI 0.01–0.19, <math>p &lt; 0.0001</math>)</p>

CPM: contralateral prophylactic mastectomy; BC: breast cancer; BBC: bilateral breast cancer; CBC: contralateral breast cancer; UBC: unilateral breast cancer; AR: absolute risk; SIR: Standardized incidence ratio; FH: family history; RD: risk difference; HR: hazard ratio.

In a retrospective study of 6230 women from high risk families, with or without a known *BRCA1/2* mutation, Rhiem observed a cumulative contralateral breast cancer risk, 25 years after a first breast cancer diagnosis of 44.1% (95% CI 37.6–50.6) in *BRCA1* positive families, 33.5% (95% CI 22.4–44.7) in *BRCA2* positive families and 17.2% (95% CI 14.5–19.9) in *BRCA1/2* negative families [56]. This effect was previously demonstrated in smaller cohort studies linking a higher contralateral breast cancer risk with a family history with and without a young age of first breast cancer diagnosis [51,53].

The age at which the affected relative is diagnosed with their first breast cancer and the presence of bilateral disease impacts on contralateral breast cancer risk. Rhiem further observes that patients diagnosed with breast cancer at age <40 years had a cumulative risk 25 years from primary diagnosis of 55.1% and 38.4% for *BRCA1* and *BRCA2*-positive family history, respectively. The corresponding risk was 28.4% in patients from non-*BRCA* families [56].

The highest risk lies with women who have relatives with early-onset, bilateral breast cancer [52–54,57]. The 10-year absolute risk in individuals whose 1st degree relative received a unilateral breast cancer diagnosis at a young age (<40 years) is similar to that of an individual with a 1st degree relative diagnosed with bilateral breast cancer (13.5% and 14.1% respectively). When there was a combination of a family history of a 1st degree relative, an affected relative with bilateral breast cancer or at a young age (<40 years), the 10-year contralateral breast cancer risk increased significantly to 36% [46]. A similar cumulative risk of contralateral breast cancer by the age of 80 (32%, 95% CI 13–66) was observed in a study of 78,775 breast cancer patients, with a maternal history of bilateral breast cancer [53].

### 5.2. Contralateral Prophylactic Mastectomy and Risk of Contralateral Breast Cancer

Contralateral prophylactic mastectomy may reduce the risk of contralateral breast cancer in women with an elevated genetic or familial risk [21]. This meta-analysis demonstrated a risk reduction in women with *BRCA*-positive families (HR 0.03;  $p = 0.0005$ ). However, only 4% (19/430) of the cohort were non-carriers, with the remaining 96% representing mutation carriers. Fayanju reported a significant reduction in pooled relative (RR 0.04, 95% CI 0.02–0.09) and absolute risk (–24%, 95% CI (–35)–(–12.4)) of metachronous contralateral breast cancer amongst recipients of contralateral prophylactic mastectomy [58]. This analysis included studies with a significant proportion of *BRCA* carriers which may lead to an overestimation of risk. A case-control study of women with stage I/II breast cancer and a positive family history reported a 95% decreased risk (HR 0.05, 95% CI 0.01–0.19;  $p < 0.0001$ ) of contralateral breast cancer following contralateral prophylactic mastectomy, at a median follow-up of 17.3 years, compared to a matched cohort of women who did not receive mastectomy. However, this cohort, with either an affected 1st or 2nd degree relative was not screened for mutation status [55].

McDonnell also demonstrated a contralateral breast cancer risk reduction following contralateral prophylactic mastectomy in pre- and postmenopausal women with a strong family history of breast/ovarian cancer i.e., 94.4% (95% CI 87.7–97.9) and 96% (95% CI 85.6–99.5) respectively, at a median follow up of 10 years using the Anderson model [59] to predict the risk [60]. Although the cohort had a strong family history, the patients had not been screened for mutations. Similar to studies with undefined gene carriers within the study population, this data should be interpreted with caution as the effect from contralateral prophylactic mastectomy may be overestimated from competing risks conferred by mutation carriers.

### 5.3. Contralateral Prophylactic Mastectomy and Survival

The evidence on the effect of contralateral prophylactic mastectomy on disease-free and overall survival is conflicting (Table 4).

A Cochrane review of 1708 women with variable familial risk, who underwent contralateral prophylactic mastectomy, concluded that although this decreased the incidence of contralateral breast cancer, there was no association with survival improvement [61]. The meta-analysis conducted



by Fayanju demonstrated no association with breast-cancer-specific and overall survival, despite a reduction in the risk of distant metastases or recurrence [58]. The lack of survival benefit from contralateral prophylactic mastectomy in breast cancer patients with elevated familial risk is also reported in smaller, retrospective cohort studies [27,62,63] but with notable exceptions. Boughey reported improved overall (HR 0.77, 95% CI 0.60–0.98;  $p = 0.03$ ) and disease-free survival (HR 0.67, 95% CI 0.54–0.84) on multivariate analysis [55]. In a review of 908 patients receiving against 46,368 not receiving contralateral prophylactic mastectomy, Herrinton demonstrated that mastectomy reduced breast cancer mortality (HR 0.57, 95% CI 0.45–0.72) and overall mortality (HR 0.6, 95% CI 0.5–0.72) across all levels of familial risk [64]. Furthermore, Davies demonstrated that young women (<40 years) with unilateral, stage I disease and a 1st degree relative with bilateral breast cancer, were the only group to have a quality-adjusted life year benefit from contralateral prophylactic mastectomy, which was similar to that of a *BRCA1/2* carrier [63].

**Table 4.** Studies looking at the impact of CPM on CBC and survival in BC patients with elevated familial risk.

Author	Year	Study Type	Follow up	Patients	Findings
Akdeniz [33]	2019	Meta-analysis	N/A	68 studies	A positive FH of BC was associated with increased CBC risk RR = 1.72 (95% CI 1.15–2.57)
Engel [16]	2019	Multicentre, prospective cohort study	Median 2.9 years	667 <i>BRCA1</i> carriers, 402 <i>BRCA2</i> carriers 1924 <i>BRCA1/2</i> noncarriers ( <i>BRCA1/2</i> -negative families)	10-year cumulative CBC risk for <i>BRCA1/2</i> non carriers 3.6% (95 CI 2.2–5.7) Women with $\geq 2$ relatives with BC had an increased risk of CBC, compared to women without any relative affected by BC HR 2.35 (95% CI 1.21–4.55) ER-negativity was not associated with an increased CBC risk in <i>BRCA1/2</i> non-carriers
Fayanju [58]	2014	Meta-analysis	N/A	14/79 studies	Patients with an elevated familial/genetic risk who had CPM (vs no CPM): Reduction in pooled RR of mCBC; RR 0.04 (95% CI 0.02–0.09; $p < 0.001$ ) Reduction in pooled AR of mCBC; RD of –24% (95% CI –35.6 to –12.4; $p = 0.013$ ) Significant reduction in rates of distant/metastatic recurrence. CPM was not associated with improved OS or BCSS
Boughey [55]	2010	Retrospective cohort	Median 17.3 years	385 patients with a positive FH; 385 matched controls (parent, sibling or 2nd degree relative with BC) * no genetic screening	Patients with stage I/II BC and a positive family history who underwent CPM had: A 95% reduction in CBC rates; adjusted HR 0.05 (95% CI 0.01–0.19, $p < 0.0001$ ) Improved OS (HR 0.77 (95% CI 0.60–0.98, $p = 0.03$ )) Improved DFS (HR 0.67 (95% CI 0.54–0.84))
McDonnell [60]	2001	Retrospective cohort	Median 10 years	745 BC patients (388 premenopausal (<50 yrs); 357 postmenopausal with a positive FH)	CPM conferred a CBC risk-reduction: In premenopausal women of 94.4% (95% CI 87.7–97.9) In postmenopausal women of 96% (95% CI 85.6–99.5)
Herrinton [64]	2005	Retrospective cohort	Median 5.7 years	1072/50,000 BC patients undergoing CPM	Across all levels of familial risk, CPM: Reduces breast cancer mortality (HR = 0.57; 95% CI 0.45–0.72) Reduces overall mortality (HR = 0.6; 95% CI 0.5–0.72)

Table 4. Cont.

Author	Year	Study Type	Follow up	Patients	Findings
Peralta [65]	2001	Retrospective cohort	Mean 6.8 years	23/64 (36%) BC patients undergoing CPM and with $\geq$ one affected 1st degree relative (not screened for mutations)	None of the patients undergoing CPM developed a subsequent CBC
Kiely [62]	2010	Retrospective cohort	Median 8 years	154/1018 women who underwent CPM, with FH of BC $\pm$ BRCA mutations	Reduced rate of CBC in women who underwent CPM with no apparent benefit in survival

CPM: contralateral prophylactic mastectomy; BC: breast cancer; CBC: contralateral breast cancer; mCBC: metachronous contralateral breast cancer; BBC: bilateral breast cancer; UBC: unilateral breast cancer; FH: Family history; RR: relative risk; AR: absolute risk; DFS: disease-free survival; OS: overall survival; BCSS: breast cancer-specific survival; ER: oestrogen receptor; HR: hazard ratio.

## 6. Young Women with Breast Cancer

### 6.1. Young Women with Breast Cancer and Contralateral Breast Cancer Risk

The definition of ‘young’ age group in the literature, varies from the “under-35”- to 50 years. Young age at first primary breast cancer diagnosis is associated with an increased contralateral breast cancer risk, poor prognosis and serves as an independent predictor of recurrence and breast-cancer-related death [66–71] (Table 5). Older studies did not account for *BRCA* mutation carriers, which may confound contralateral breast cancer risk and survival. Furthermore, they do not consider risk-reducing adjuvant therapies. In a retrospective study of 652 patients  $\leq$ 35 years compared to 2608 women  $>$ 35 years, the relative risk of contralateral breast cancer was 2.48 in the younger, compared to the older group [70]. This finding is supported by Li, who demonstrated an increased HR of 2.8 (95% CI 1.1–6.9), 2.1 (95% CI 1.1–4.4) and 1.9 (95% CI 1.1–3.5) in the  $\leq$ 29 years, 30–34 years and 35–39 years age groups, compared to women diagnosed at age  $\geq$ 40 [67]. The contralateral breast cancer risk is further elevated in HER2-overexpressing and triple negative subtypes [70].

Table 5. Studies looking at the impact of CPM on CBC and survival in young women with breast cancer.

Author	Year	Study Type	Median Follow up	Patient Demographics (Age, CPM Status)	Findings
Chen [2]	2019	Retrospective cohort	113 months	<35 years and CPM 811/3083 (26.3%) 35–39 years and CPM 1243/5961 (20.9%)	No difference in BCSS from CPM HR 1.209 (95% CI 0.908–1.610, $p = 0.194$ ) No difference in OS from CPM HR 1.179 (95% CI 0.902–1.540, $p = 0.228$ )
Yu [72]	2018	Retrospective cohort	6.9 years	910/1806 young patients (18–50 years) with CPM	No difference in OS in women with a young age (18–50 years) who had CPM HR 0.93 (95% CI 0.70–1.24; $p = 0.627$ )
Pesce [73]	2014	Retrospective cohort	6.1 years	4338/10,289 (29.7%) young women (<45 years) with Stage I/II cancer with CPM	CPM provides no survival benefit in young women (<45 years) Compared to unilateral mastectomy HR 0.93; $p = 0.39$ With early-stage (T1N0) breast cancer HR 0.85; $p = 0.37$ With ER-negative breast cancer HR 1.12; $p = 0.32$
Bedrosian [74]	2010	Population based cohort study	47 months	3731/27,336 (13.6%) young women (18–49 years) with CPM	CPM offers benefit in BCSS for young women (18–49 years) with early stage, ER-negative breast cancer HR 0.68 (95% CI 0.53–0.69), $p < 0.001$

Table 5. Cont.

Author	Year	Study Type	Median Follow up	Patient Demographics (Age, CPM Status)	Findings
Bouchard-Fortier [75]	2018	Population-based cohort	11 years	81/614 (13.2%) young women ( $\leq 35$ years) with CPM	Risk of recurrence (breast/distant) was lower in the CPM group HR 0.61, $p = 0.02$ No difference in breast cancer-specific mortality from CPM HR 0.73 (95% CI 0.47–1.21)
Zeichner [71]	2014	Retrospective cohort	68 months	42/481 (8.73%) young women ( $< 40$ years) with CPM	CPM provides a benefit in 10-year overall survival * HR 2.35 (95% CI 1.02–5.41, $p = 0.046$ ) * effect not seen at 5-year overall survival
Lazow [76]	2018	Population-based cohort	Mean 62 months	4139/11,859 (34.9%) young women ( $< 40$ years) with CPM	CPM improves 10-year overall survival HR 0.75 (95% CI, 0.59–0.96) $p = 0.023$
Park [77]	2017	Population based, national database study	Not stated	3648 DCIS patients $< 40$ years (25.8% UM; 15.8% CPM)	No overall survival benefit from CPM compared to UM in the $< 40$ years group

OS: overall survival; CPM: contralateral prophylactic mastectomy; unilateral mastectomy: BCSS: breast-cancer-specific survival.

### 6.2. Contralateral Prophylactic Mastectomy and Risk of Contralateral Breast Cancer

The younger age group is generally underrepresented in studies evaluating the role of contralateral prophylactic mastectomy. Using a Surveillance, Epidemiology, End Results database analysis of 107,106 women, of whom 8902 (8.3%) underwent contralateral prophylactic mastectomy, Bedrosian conducted a subgroup analysis of young women ( $< 50$  years) and the risk of contralateral breast cancer after contralateral prophylactic mastectomy, in both ER-negative and ER-positive, early-stage breast cancer. In ER-positive disease, the cumulative incidence of contralateral breast cancer during the 6-year study period was 0.13% vs. 0.46% ( $p = 0.07$ ) in the contralateral prophylactic mastectomy vs. non-mastectomy group, and in ER-negative disease, 0.16% vs. 0.90% ( $p = 0.05$ ) respectively [74]. These results should be interpreted with caution though as the study population was not screened for genetic carriers and also patients with a strong family history were not excluded.

### 6.3. Contralateral Prophylactic Mastectomy and Survival

There is conflicting data on the impact of contralateral prophylactic mastectomy on survival in this patient group. In a population-based study of 614 women  $< 35$  years, 81 (13.2%) of whom were elected for contralateral prophylactic mastectomy, Bouchard-Fortier demonstrated that recurrences, defined as local, regional or distant, were significantly fewer for patients with contralateral prophylactic mastectomy than without (32.1% vs. 52.9%,  $p < 0.001$ ; HR 0.61;  $p = 0.02$ ). However, this did not translate to an improvement in breast cancer-specific survival [75].

In an analysis of the National Cancer Database between 2004 and 2014, Lazow demonstrated that after controlling for patient demographics, tumor grade and use of adjuvant therapies, bilateral mastectomy in women  $< 40$  years was associated with increased 10-year overall survival (HR 0.75, 95% CI 0.59–0.96;  $p = 0.023$ ), compared to the unilateral mastectomy group [76]. This trend was also observed in a preceding National Cancer Database review from 1998–2002, demonstrating a 5-year overall survival benefit of 2% in young patients (adjusted HR 0.88, 95% CI 0.83–0.93;  $p < 0.001$ ) between these two groups [78]. In a retrospective study of 42/481 (8.73%) young women  $< 40$  years, who were elected for contralateral prophylactic mastectomy, Zeicher reported that this was associated with improved 10-year overall survival (HR 2.35, 95% CI 1.02–5.41;  $p = 0.046$ ), although this effect was not demonstrable for 5-year overall survival [71].

There is a suggestion that contralateral prophylactic mastectomy may confer benefit in young women with early-stage, ER-negative breast cancer. In a population-based study of 107,106 breast cancer patients, 3731 (3.48%) of whom were young (18–49 years), contralateral prophylactic mastectomy

was associated with improved disease-specific mortality (HR 0.68, 95% CI 0.53–0.88;  $p = 0.004$ ). This effect was not reproduced in young women with early-stage, ER-positive breast cancer [74].

Other retrospective cohort or population-based studies refute the survival benefit of contralateral prophylactic mastectomy in young women [72,73,75,77]. In a review of 9044 young women (<40 years) with breast cancer, Chen demonstrated no improvement in overall or breast cancer-specific survival [2]. This was supported in a retrospective study of 10,226 patients with invasive lobular carcinoma, demonstrating no overall survival benefit from contralateral prophylactic mastectomy in the 18–50 years group, at a median follow up of 6.9 years [72]. Moreover, in a review of 14,627 women and at median follow-up of 6.1 years, having matched for tumour size/grade, ER status and nodal status, Pesce demonstrated that contralateral prophylactic mastectomy offered no overall survival benefit, in women aged <45 years, with stage I/II breast cancer (HR 0.93,  $p = 0.39$ ) [73].

Overall, these findings should be interpreted with caution as the quality of the data does not allow for definitive conclusions to be drawn.

## 7. Discussion

Contralateral prophylactic mastectomy is increasingly being performed despite an ambiguity of evidence to support an oncological benefit. In 2007 and 2009, two studies reported that contralateral prophylactic mastectomy rate had increased 148% and 150% among all patients diagnosed with non-invasive and invasive breast cancer respectively [79,80]. Current trends in the U.S.A show an absolute percentage increase in the range of 25% [81]. This trend is modest in European studies suggesting a difference in practice and healthcare environments [82–84]. Nonetheless, this increased utilization of contralateral prophylactic mastectomy is a cause of concern for clinicians because of the associated surgical risks, complications, and psychological and financial burden in the absence of robust evidence to support significant oncological benefits.

Although intuitively it is expected that contralateral prophylactic mastectomy would decrease the risk of contralateral breast cancer, the available data only support this in patients with *BRCA1/2* gene mutations [14,26,27]. In women with strong family history or young age at diagnosis, the effect of contralateral prophylactic mastectomy is less well studied and the existing literature should be interpreted with caution because of the potential biases. At present, there are no models that allow for calculation of contralateral breast cancer risk in a polyfactorial model. Such a model might be useful in stratifying risk and aiding physicians to provide precise and unbiased estimation of risk, in order to offer individualized counselling to patients, inform decision-making and mitigate patient overestimation of cancer risk which may drive unnecessary surgery.

Despite the potential decrease in contralateral breast cancer, the effect of contralateral prophylactic mastectomy on oncological outcomes is debatable as studies suggest that this reduction is not translated into survival benefit. Moreover, the role of contralateral prophylactic mastectomy per se as a contributing factor for improved outcomes in women with unilateral breast cancer is difficult to accurately define, as the majority of the available data is of limited quality. Meta-analyses are only as strong as the independent studies they comprise. The majority of studies are retrospective cohorts and based on population/family studies. Therefore, the results should be interpreted with caution because of potential uncontrolled biases. The way to address these issues is with higher quality data but it is unlikely that the future will harbour randomized clinical trials investigating the impact of contralateral prophylactic mastectomy on contralateral breast cancer risk and survival due to patient preference and ethical considerations. One proposal is to set up robust prospective registries to help enhance our knowledge in the field. The majority of existing studies do not account for the significant role conferred by improved systemic therapies and its effect on contralateral breast cancer risk and improved oncological outcomes, factors that merit future research.

Recently, and in order to aid clinicians approach this controversial topic, both the American Society of Breast Surgeons and Association of Breast Surgery published consensus statements on the utilization of contralateral prophylactic mastectomy. Both were aligned on supporting its use in women with

significant contralateral breast cancer risk i.e., *BRCA1/2* mutations, patients with a history of mantle field radiation to the chest before age 30 years [85,86]. However, a multidisciplinary, individualised approach is required to help women in their informed decision-making process.

## 8. Conclusions

In conclusion, contralateral prophylactic mastectomy may be supported in ‘high-risk’ groups as evidence indicates a possible reduction in contralateral breast cancer risk and also potentially improved oncological outcomes. The evidence to demonstrate that this may confer benefit in the other risk groups or in older patients is less established. It is therefore imperative to follow a multidisciplinary, personalised approach, to educate women on the best available evidence and to support individuals in a shared-decision making process.

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Article

# *APOBEC3B* Gene Expression in Ductal Carcinoma In Situ and Synchronous Invasive Breast Cancer

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**Abstract:** The underlying mechanism of the progression of ductal carcinoma in situ (DCIS), a non-obligate precursor of invasive breast cancer (IBC), has yet to be elucidated. In IBC, Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like 3B (*APOBEC3B*) is upregulated in a substantial proportion of cases and is associated with higher mutational load and poor prognosis. However, *APOBEC3B* expression has never been studied in DCIS. We performed mRNA expression analysis of *APOBEC3B* in synchronous DCIS and IBC and surrounding normal cells. RNA was obtained from 53 patients. The tumors were categorized based on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2) and phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA) mutation status. *APOBEC3B* mRNA levels were measured by RT-qPCR. The expression levels of paired DCIS and adjacent IBC were compared, including subgroup analyses. The normal cells expressed the lowest levels of *APOBEC3B*. No differences in expression were found between DCIS and IBC. Subgroup analysis showed that *APOBEC3B* was the highest in the ER subgroups of DCIS and IBC. While there was no difference in *APOBEC3B* between wild-type versus mutated PIK3CA DCIS, *APOBEC3B* was higher in wild-type versus PIK3CA-mutated IBC. In summary, our data show that *APOBEC3B* is already upregulated in DCIS. This suggests that *APOBEC3B* could already play a role in early carcinogenesis. Since *APOBEC3B* is a gain-of-function mutagenic enzyme, patients could benefit from the therapeutic targeting of *APOBEC3B* in the early non-invasive stage of breast cancer.

**Keywords:** *APOBEC3B*; gene expression; breast cancer; ductal carcinoma in situ; infiltrating breast cancer; PIK3CA

## 1. Introduction

Ductal carcinoma in situ (DCIS) is a non-obligate precursor of invasive breast cancer (IBC) [1]. This is supported by previous studies that reported a high genomic concordance of synchronous DCIS and IBC [2–4]. However, despite molecular similarities, recent in-depth genetic studies also reported specific mutations that were either restricted to the in situ or the invasive component [3,5]. Increased insight in the molecular changes during DCIS progression has the potential to reveal novel, potentially targetable drivers of progression.

A major role of Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like 3B (*APOBEC3B*) has been reported in breast cancer and several other cancers [6–9]. This enzyme is a member of the *APOBEC* family of deaminases and is involved in DNA cytosine deaminase activity,

which has diverse biological functions, including activities in the innate immune system by restricting virus replication [10]. The upregulation of APOBEC3B is correlated with increased C-to-T transitions and increased mutational load, including known driver mutations in PIK3CA and tumor protein 53 (TP53) [10–12]. *APOBEC3B* mRNA is upregulated in a substantial proportion of IBC cases and an association with poor clinical outcome has been reported in Estrogen receptor (ER)-positive subtypes [13]. In addition, we recently reported higher mRNA levels of *APOBEC3B* in breast cancer metastasis as compared to the corresponding primary tumor, which implied that breast cancer progression is associated with the upregulation of APOBEC3B [14].

In this study we investigated *APOBEC3B* mRNA expression levels in synchronous DCIS and IBC and correlated the expression with PIK3CA mutation status in order to increase our understanding regarding the expression levels of this enzyme during progression from the in situ to the invasive stage. We believe this could improve breast cancer care in the future since APOBEC3B is a gain-of-function mutagenic enzyme, so patients could potentially be treated with small molecules at a very early, non-invasive stage.

## 2. Results

### 2.1. General Clinicopathological Data

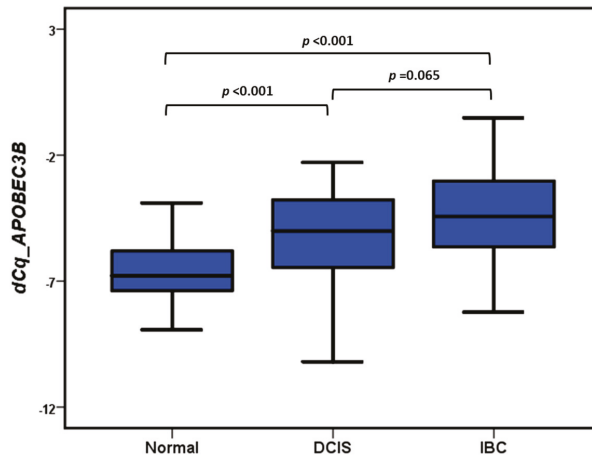
In total, 53 patients were included. Table 1 provides an overview of the clinicopathological data of all patients. The overall median age was 53 years (range 28–102 years). The majority of DCIS and IBC samples were high grade (62.3 and 54.7%, respectively). There was no difference in grade between DCIS and adjacent IBC (Fisher Exact Probability Test  $p = 0.92$ ). Based on immunohistochemical staining, IBCs were categorized into the following five breast cancer subtype categories: ER+/PR high/Her2– ( $n = 13$ ), ER+/PR– or low/Her2– ( $n = 12$ ), ER+/any PR/Her2+ ( $n = 11$ ), ER–/PR–/Her2+ ( $n = 8$ ), or ER–/PR–/Her2– ( $n = 9$ ).

**Table 1.** Clinicopathological features of patients with ductal carcinoma in situ (DCIS) and adjacent invasive breast cancer (IBC) ( $n = 53$ ).

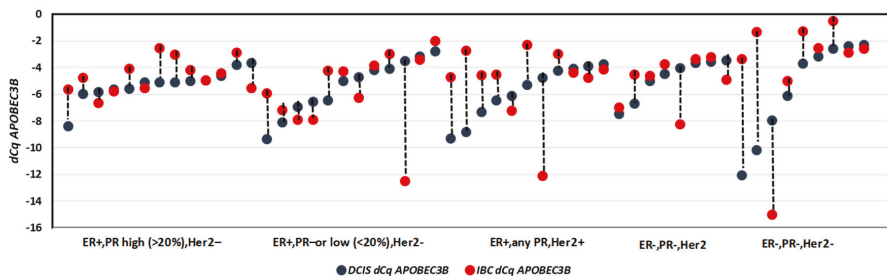
Characteristic	<i>n</i>	(%)
<b>Age at diagnosis</b>	53	
years, median (range)	(28–102)	
<b>Type of surgery</b>		
Breast-conserving surgery	24	45.3
Mastectomy	29	54.7
<b>Grade DCIS</b>		
1	1	49.1
2	19	39.6
3	33	7.5
<b>Grade IBC</b>		
1	1	49.1
2	21	39.6
3	31	7.5
<b>Tumor size</b>		
≤2 cm	28	49.1
>2–5 cm	21	39.6
>5 cm	4	7.5
Missing	0	3.8
<b>Subtypes based on immunohistochemistry</b>		
ER+/PR high/Her2–	13	24.5
ER+/PR– or low/Her2–	12	22.6
ER+/any PR/Her2+	11	20.8
ER–/PR–/Her2+	8	15.1
ER–/PR–/Her2–	9	17.0

## 2.2. APOBEC3B Expression in Synchronous Normal, DCIS and IBC Cells

Both the Kruskal-Wallis Test and the Median Test indicated that there was a significant difference ( $p < 0.001$ ) in APOBEC3B mRNA levels between the normal controls, DCIS and IBC. APOBEC3B mRNA was lower expressed in the normal mammary epithelial tissue adjacent DCIS and IBC (unpaired Mann-Whitney  $U$  Test and paired Wilcoxon Signed Ranks Test  $p < 0.001$ ) (Figure 1). There was no statistically significant difference in APOBEC3B mRNA expression between DCIS and IBC (unpaired Mann-Whitney  $U$  Test  $p = 0.065$  (Figure 1), Wilcoxon Signed Ranks Test  $p = 0.082$ ). (Figure 2).



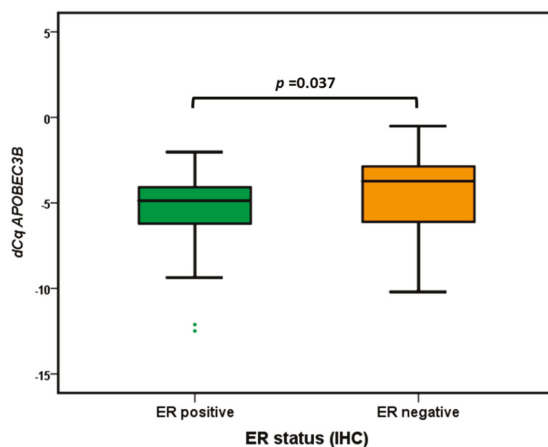
**Figure 1.** Boxplots of APOBEC3B mRNA expression levels in paired normal, DCIS and IBC ( $n = 53$ ). Differences between normal, DCIS and IBC were analyzed by the Mann-Whitney  $U$  test.



**Figure 2.** APOBEC3B expression levels in paired DCIS and IBC ( $n = 53$ ). Wilcoxon Signed Ranks Test  $p = 0.082$ .

## 2.3. APOBEC3B mRNA Subgroup Analysis

Previous studies reported elevated APOBEC3B mRNA levels in breast cancers with otherwise aggressive characteristics, including high histological grade and lack of estrogen expression [7,13,15]. For both DCIS and IBC, there was no correlation between APOBEC3B expression levels and tumor diameter (Spearman Rank Correlation Test  $p > 0.05$ ) or histological grade (Kruskal-Wallis Test  $p > 0.05$ ). Our breast cancer subtype analysis showed that the expression of APOBEC3B was the highest in the ER- subgroup (Mann-Whitney  $U$  Test,  $p = 0.037$ ) (Figure 3).



**Figure 3.** Boxplots of Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like 3B (APOBEC3B) mRNA expression levels according to ER status. The difference between ER+ and ER- cases was analyzed by the Mann-Whitney *U* test.

#### 2.4. APOBEC3B Expression in Epithelial Versus Inflammatory Cells

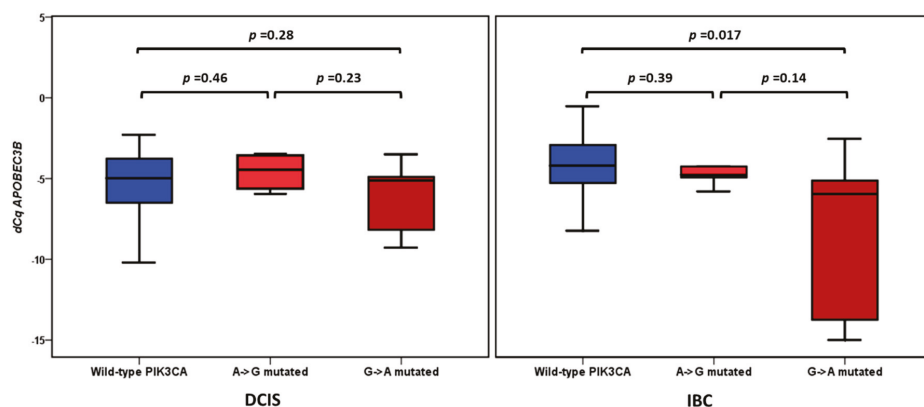
Based on the positive correlation between APOBEC3B and marker for epithelial content (EPCAM) mRNA levels (Spearman Rank Correlation test,  $p = 0.005$  for DCIS,  $p = 0.001$  for IBC), APOBEC3B mRNA was mostly expressed by epithelial cells. Of note, there was no significant difference in the levels of EPCAM mRNA between DCIS and synchronous IBC (Wilcoxon Signed Ranks Test,  $p = 0.18$ ).

Since inflammatory cells also express APOBEC3B [16], we investigated whether the number of inflammatory cells could have biased our results by comparing Protein Tyrosine Phosphatase Receptor Type C (PTPRC, gene for the common leukocyte antigen CD45) mRNA levels from DCIS and IBC. There was no correlation between APOBEC3B and PTPRC mRNA levels (Spearman Rank Correlation test,  $p = 0.18$  for DCIS and  $p = 0.29$  for IBC). However, IBC expressed slightly higher levels of PTPRC when compared with DCIS (Wilcoxon Signed Ranks Test,  $p = 0.023$ ).

#### 2.5. APOBEC3B Expression and PIK3CA Mutation Status

In a recently published study [17], we detected a PIK3CA somatic hotspot mutation in 24.7% (18 out of 73) patients. For these 18 PIK3CA-positive patients, a significantly higher PIK3CA variant allele frequency (VAF) was detected in the DCIS component (45.8%) when compared with the synchronous IBC component (31.7%) ( $p = 0.007$ ). For the  $n = 14$  PIK3CA mutation-positive patients (26.4%) included in the current study, a significantly higher PIK3CA VAF was also detected in the DCIS component (52.3%) when compared with the synchronous IBC component (37.2%) ( $p = 0.027$ ). The correlation of PIK3CA VAF with APOBEC3B showed a negative Spearman Rank correlation in IBC ( $r_s = -0.33$ ,  $p = 0.001$ ,  $n = 53$ ). For the DCIS cases, there was no such correlation ( $r_s = 0.02$ ,  $p = 0.89$ ,  $n = 53$ ). Analyzing these data irrespective of the degree of the PIK3CA VAF levels revealed that for the 53 patients analyzed in this study, APOBEC3B mRNA levels in IBC were significantly lower in the eight patients with exon 9 (G to A)-mutated PIK3CA when compared with the  $n = 39$  wild-type PIK3CA cases (Mann-Whitney *U* test  $p = 0.017$ ). No such difference was observed for the DCIS cases ( $p = 0.28$ ) (Figure 4). Albeit not statistically significant, APOBEC3B mRNA levels were higher overall in the  $n = 39$  PIK3CA wild-type IBC samples when compared with the PIK3CA wild-type DCIS samples (Mean  $\pm$  SEM:  $-4.54 \pm 0.36$  for IBC versus  $-5.38 \pm 0.35$  for DCIS) and lower in the  $n = 8$  G-to-A PIK3CA-mutated IBC samples when compared with G-to-A PIK3CA-mutated DCIS samples (Mean  $\pm$  SEM:  $-6.52 \pm 1.66$  for IBC versus  $-6.14 \pm 0.74$  for DCIS). Although the majority of samples with a PIK3CA mutation were

ER+, there was no significant interaction effect between ER status and the absence or presence of the two types of tested PIK3CA mutations ( $p = 0.46$  for DCIS and  $p = 0.20$  for IBC).



**Figure 4.** Boxplots of *APOBEC3B* mRNA expression levels according to PIK3CA mutation status. The differences between wild-type (blue boxes) and mutated (red boxes) PIK3CA cases were analyzed by the Mann-Whitney *U* test.

### 3. Discussion

*APOBEC3B* has been identified as an important factor in the evolution of breast cancer [8]. In a recently published pan-tissue, pan-cancer analysis of RNA-seq data specific to the seven *APOBEC3* genes in 8951 tumors, 786 cancer cell lines and 6119 normal tissues, *APOBEC3B* consistently demonstrated its association with proliferative cells and processes, in contrast to other *APOBEC3s*, especially *APOBEC3G* and *APOBEC3H*, which were revealed as more immune cell related [9]. Our current data showed that *APOBEC3B* mRNA is already upregulated in the in situ stage of breast cancer, which is in line with the high genomic resemblances between DCIS and IBC [18]. In a study we performed earlier, we observed higher mRNA levels of *APOBEC3B* in breast cancer metastasis as compared to the corresponding primary tumor [14], supporting our hypothesis that, already starting from DCIS, breast cancer progression is associated with deregulated expression of *APOBEC3B*.

Tumors with upregulated *APOBEC3B* demonstrate a higher mutational load, which could explain the aggressive behavior of these tumors [7,12]. Two hotspot G-to-A mutations in exon 9 of the—often mutated in breast cancer—PIK3CA gene (E542K and E545K) are thought to be generated by *APOBEC3B* induced C-to-T (G-to-A) transitions [11]. Whether *APOBEC3B* is still needed once the mutations are present needs further investigation. In the study of Kosumi et al., *APOBEC3B* expression in esophageal squamous cell carcinoma was significantly correlated with PIK3CA mutations in exon 9 [10]. However, no correlation was found between *APOBEC3B* expression and PIK3CA mutations status in a Japanese breast cancer cohort [15]. Although PIK3CA mutations are known to be more prevalent in ER+ cases [19], and thus might have been a confounder in our analysis, we found no significant difference in the distribution of wild-type and mutated PIK3CA in ER+ and ER− cases. In our cohort, *APOBEC3B* levels were decreased in specifically the G-to-A PIK3CA-mutated IBC samples when compared with wild-type PIK3CA IBC tumors. In the synchronous DCIS counterpart, however, there was no difference in *APOBEC3B* levels between mutated and PIK3CA wild-type tumors. This might suggest that, in contrast to DCIS, the invasive tumors no longer need *APOBEC3B* to proliferate and metastasize. Previous studies reported elevated *APOBEC3B* mRNA levels in breast cancers with otherwise aggressive characteristics, including high histological grade and lack of estrogen expression [7,13,15]. This is consistent with our subgroup analysis, which showed higher *APOBEC3B* levels in synchronous DCIS and IBC of ER− tumors as compared to ER+ tumors. However, in our



study, no significant correlations were found between *APOBEC3B* levels and histological grade and/or tumor diameter. This could be due to the fact that the majority of our samples were high grade.

This is the first study evaluating *APOBEC3B* levels within DCIS and co-existing IBC, including different breast cancer subtypes. However, our study has several limitations, such as the relatively small size of our cohort and the analysis of a limited mRNA panel only, with the main focus on *APOBEC3B*. Since upregulated *APOBEC3B* is associated with higher mutational load, evaluation of the mutational status of additional markers besides *PIK3CA* will be interesting. Another limitation is that *APOBEC3B* is also expressed by inflammatory cells, which could have influenced our data because we performed manual microdissection, and thus contamination with inflammatory cells was not completely avoidable. Although IBC expressed slightly higher levels of *PTPRC* (the gene for leukocyte antigen CD45) than DCIS, there was no correlation between *APOBEC3B* and *PTPRC* mRNA levels. Based on this analysis, it seems unlikely that the number of inflammatory cells biased our data.

Increased insight in molecular mechanisms that contribute to DCIS progression will improve the development of a personalized treatment strategy for patients with DCIS. *APOBEC3B* could be a potential therapeutic target since it is non-essential, but it has an active enzymatic activity that may be inhibited [7]. Patients with DCIS could therefore benefit from such therapeutic molecules by inhibiting tumor evolution. Concept inhibitors have already been developed for the related enzyme *APOBEC3G* [20,21]. Additional clinical and pharmaceutical assays are necessary to develop and explore the potential benefit of *APOBEC3B* inhibitors.

## 4. Materials and Methods

### 4.1. Patient Materials

Fifty-three patients with synchronous DCIS and IBC were enrolled. We used coded leftover patient material in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (<http://www.federa.org/codes-conduct>). This article is approved by the Medical Ethics Committee of the Erasmus MC (approval number MEC 02.953). According to national guidelines, no informed consent was needed for this study.

Formalin-fixed-paraffin-embedded (FFPE) hematoxylin and eosin (H&E)-stained whole sections of excision specimens were collected and reviewed by two pathologists (Carolien H. M. van Deurzen and Shusma C. Doebar). Histopathological features included the grade of IBC [22], IBC diameter, ER, PR and Her2 status, and grade of DCIS [23]. Tumors were divided into subtypes based on immunohistochemistry (ER, PR and Her2), including the following 5 categories: ER+/PR high/Her2−; ER+/PR− or low/Her2−; ER+/any PR/Her2+; ER−/PR−/Her2+; ER−/PR−/Her2−. ER was considered positive when at least 10% of the tumor cells were positive, irrespective of intensity, according to national guidelines (<https://richtlijnendatabase.nl>). Low PR was defined as ≤ 20% [24]. Immunohistochemical HER2 expression was scored according to international guidelines [25]. Equivocal cases were evaluated by silver in situ hybridization.

### 4.2. RT-qPCR

RNA was extracted from tissue areas composed of at least 50% IBC or DCIS cells and analyzed by RT-qPCR as described before [14,17]. In brief, these cells were obtained by microdissection from FFPE tissue, which was performed with a sterile needle under a stereomicroscope. RNA was extracted from these cells using the Qiagen (Hamburg, Germany) AllPrep DNA/RNA FFPE Kit according to the manufacturer's instructions. RNA concentrations were measured with a Nanodrop 2000 system. cDNA was generated from 50 ng/μL cDNA and was generated for 30 min at 48 °C with the RevertAid H minus kit (Thermo Fisher Scientific, Breda, The Netherlands) and gene-specific pre-amplified with Taqman PreAmp Master mix (Thermo Fisher Scientific) for 15 cycles, followed by Taqman probe based real-time PCR according to the manufacturer's instructions in a MX3000P Real-Time PCR System (Agilent, Amsterdam, The Netherlands). The following intron-spanning gene expression assays (all from Thermo

Fisher Scientific) were evaluated: *APOBEC3B*, assay ID: hs00358981\_m1; *EPCAM*, hs00158980\_m1, and *PTPRC*, hs00236304\_m1. Messenger RNA levels were quantified relative to the average expression of 2 reference genes (*GUSB*, hs9999908\_m1 and *HMBS*, hs00609297\_m1) using the delta Cq (average Cq reference genes – Cq target gene) method. According to GeNorm and NormFinder, the average of these two reference genes was the most stable expressed across our samples (M-value = 0.59, SD = 0.29). Also, when taking the different groups into account, the inter and intra variation was the lowest for the average of our 2 reference genes (SD = 0.19 for the NormFinder analysis across the control, DCIS and IBC groups and SD = 0.24 for the NormFinder analysis across the ER/PR/Her2 groups). Samples with an average reference gene expression of Cq > 25 were considered to be of insufficient RNA quality and excluded from further analysis, together with their paired samples. A serially diluted RNA pool of FFPE breast tumor samples was included in each experiment to evaluate the linear amplification and efficiencies for all genes included in the panel and absence of amplification in the absence of reverse transcriptase. All gene transcripts were equally efficient amplified (range 94–106%) and were negative in the absence of reverse transcriptase. A summary of the performance of our assays on these serially diluted samples is shown in Supplementary Table S1.

#### 4.3. PIK3CA Mutation Status

PIK3CA mutation status and VAFs were measured as described before [17]. In brief, DNA was extracted from the same micro-dissected FFPE tissues used for RNA extraction using the Qiagen (Hamburg, Germany) AllPrep DNA/RNA FFPE Kit. The SNaPshot Multiplex System for SNP Genotyping (Thermo Fisher Scientific) was used to identify samples positive for PIK3CA hotspot mutations in exon 9 and exon 20. Next, we used digital PCR (dPCR) to validate the SNaPshot results and quantify the relative number of PIK3CA-mutated copies (of E542K, E545K in exon 9 and H1047R and H1047L in exon 20) in both the DCIS and IBC component of those patients with a PIK3CA mutation identified by SNaPshot analysis.

#### 4.4. Statistical Analyses

GeNorm and NormFinder [26,27], present in GenEx qPCR data analysis software (version 6.1, MultiD, Göteborg, Sweden), were used to assess the stability of our reference genes. SPSS version 24 was used for the statistical analyses. Because our *APOBEC3B* mRNA data were not normally distributed (skewness  $-1.01 \pm 0.33$  and  $-1.75 \pm 0.33$ , kurtosis  $0.80 \pm 0.64$  and  $3.60 \pm 0.64$  for DCIS and IBC, respectively), we only used non-parametric tests. The Wilcoxon Signed Ranks Test was used to compare levels in paired DCIS and IBC and unpaired analyses were performed using the Wilcoxon or Mann-Whitney *U* Test or the Fisher Exact Probability Test for contingency table data. Continuous variables were analyzed by the Spearman Rank Correlation test. *p*-values  $\leq 0.05$  were considered statistically significant.

### 5. Conclusions

In conclusion, our results indicate that *APOBEC3B* mRNA is similarly upregulated in DCIS and IBC, but declines in PIK3CA-mutated IBC, which suggests that *APOBEC3B* plays a role in the early stages of breast carcinogenesis. Since *APOBEC3B* is a gain-of-function mutagenic enzyme, it could be a candidate for therapeutic targeting in an early, non-invasive stage of breast cancer.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/11/8/1062/s1>, Table S1: Performance of the Taqman mRNA assays used in this study.

**Author Contributions:** Conceptualization, A.M.S., S.C.D. and C.H.M.v.D. Methodology, A.M.S., S.C.D., E.I.V., V.d.W., C.M.B., M.C.A. and C.H.M.v.D.; Formal analysis, A.M.S.; Validation, S.C.D., C.H.M.v.D. Writing—Original Draft Preparation, A.M.S. and S.C.D.; Writing—Review and Editing, A.M.S., S.C.D., J.M.W.M. and C.H.M.v.D.; Visualization, A.M.S.; Supervision, J.W.M.M. and C.H.M.v.D. All co-authors have read and approved of the manuscript and acknowledge their contributions.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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Article

# Why Has Breast Cancer Screening Failed to Decrease the Incidence of de Novo Stage IV Disease?

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**Abstract:** *Background:* Despite screening mammography, the incidence of Stage IV breast cancer (BC) at diagnosis has not decreased over the past four decades. We previously found that many BCs are small due to favorable biology rather than early detection. This study compared the biology of Stage IV cancers with that of small cancers typically found by screening. *Methods:* Trends in the incidence of localized, regional, and distant female BC were compared using SEER\*Stat. The National Cancer Database (NCDB) was then queried for invasive cancers from 2010 to 2015, and patient/disease variables were compared across stages. Biological variables including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2), grade, and lymphovascular invasion were sorted into 48 combinations, from which three biological subtypes emerged: indolent, intermediate, and aggressive. The distributions of the subtypes were compared across disease stages. Multivariable regression assessed the association between Stage IV disease and biology. *Results:* SEER\*Stat confirmed that the incidence of distant BC increased between 1973 and 2015 (annual percent change [APC] = 0.46). NCDB data on roughly 993,000 individuals showed that Stage IV disease at presentation is more common in young, black, uninsured women with low income/education and large, biologically aggressive tumors. The distribution of tumor biology varied by stage, with Stage IV disease including 37.6% aggressive and 6.0% indolent tumors, versus sub-centimeter Stage I disease that included 5.1% aggressive and 40.6% indolent tumors ( $p < 0.001$ ). The odds of Stage IV disease presentation more than tripled for patients with aggressive tumors (OR3.2, 95% CI 3.0–3.5). *Conclusions:* Stage I and Stage IV breast cancers represent very different populations of biologic tumor types. This may explain why the incidence of Stage IV cancer has not decreased with screening.

**Keywords:** breast cancer; stage IV; incidence; tumor biology; NCDB; SEER

## 1. Introduction

Despite widespread breast cancer screening in the United States, the incidence of de novo Stage IV breast cancer has not decreased. Esserman and colleagues called attention to this irregularity in 2009, showing that localized breast cancer incidence surged with the introduction of disease screening in the 1980s, without a corresponding decrease in distant disease [1]. Works by Bleyer and colleagues have reinforced the conclusion that screening is not meaningfully lowering the incidence of advanced disease [2,3].

The reasons underlying this problem are still unknown. Esserman presciently cited tumor biological factors as likely determinants of disease screenability, calling for the incorporation of such factors into screening and treatment guidelines [1]. Recently, Lannin and Wang showed that small tumors—the majority of which are found on mammography screening—have a distinctly favorable biological profile that dictates an indolent growth pattern [4]. Along similar lines, we hypothesized that de novo Stage IV breast cancer may have a uniquely aggressive biology, granting it growth properties that allow it to escape detection by screening. The purpose of this study was to compare the tumor biology of de novo Stage IV breast cancer with that of small cancers typically detected by screening mammography.

## 2. Methods

### 2.1. Data and Patient Selection

Data for this study were drawn from both the Surveillance, Epidemiology, and End Results database (SEER, November 2017 submission) and the National Cancer Database (NCDB 2015 Participant User File, downloaded 15 December 2017). The original SEER 9 registry data, spanning 1973–2015, were used to analyze long-term population-based incidence trends of the various disease stages. SEER was chosen over NCDB for this analysis, since it contains many more years of incidence data and is population-based and age-adjusted.

NCDB data from 2010 to 2015 were used to explore patient and disease characteristics of Stage IV disease in a large modern population, as well as to compare the tumor biological profiles of Stages I–IV. NCDB was chosen over SEER for this analysis since it contains more robust data on disease characteristics and captures a larger population of breast cancer patients in the United States. The NCDB is a joint project of the Commission on Cancer of the American College of Surgeons and the American Cancer Society. The data used in this study were derived from a de-identified NCDB file. The American College of Surgeons and the Commission on Cancer have not verified and are not responsible for the analytic or statistical methodology employed nor for the conclusions drawn from these data by the investigators.

Included in this study were female patients with invasive breast cancer with known disease stage (in situ and American Joint Committee on Cancer [AJCC] Stage 0 excluded). The analyses of the incidence and patient/disease factors were conducted using targeted statistical methods and software, as outlined below.

### 2.2. Incidence

To identify long-term breast cancer incidence trends by disease stage, the SEER 9 registry data were queried for cases of localized, regional, and distant breast cancer (SEER Historic Stage A variable) from 1973 to 2015. The Historic Stage A variable is traditionally used for analyses prior to 1988, when recoding for AJCC stage was unavailable in SEER. Localized disease includes cancer confined to the breast. Regional disease refers to contiguous organ spread, including regional lymph nodes and the chest wall. Distant disease denotes remote organ metastasis detected at the time of diagnosis. SEER\*Stat software (Version 8.3.5, accessed on 20 April 2018) was used to calculate population-based incidence rates and annual percent change (APC). The two-sided *p*-values were set at <0.05.

### 2.3. Patient and Tumor Characteristics

The NCDB was queried for cases from 2010 to 2015 of “NCDB Analytic Stages I–IV,” which uses the AJCC 7th edition pathologic stage classification to collapse sub-stages into their broader designations. Stages I–III cases were consolidated and compared with Stage IV in the univariable analysis of patient characteristics, including race/ethnicity, age, insurance, median household income, and education level, as well as disease characteristics, including histology, tumor size, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor 2 status (Her2),

grade, and lymphovascular invasion (LVI). Nodal status was not included in this analysis, as debate exists as to whether lymph node spread marks biological predisposition versus a tumor’s natural history when left untreated. All patients with known disease stage, including those with other missing variables, were included in this analysis, totaling 992,687 patients. Chi-squared testing was used to detect differences in patient and disease variables between Stage IV and non-Stage IV cancer, with statistical significance set at  $p < 0.05$ .

Five markers of biological activity reported in the NCDB were found to be associated with Stage IV in the univariable analysis: ER, PR, Her2, grade, and LVI. Their values were recombined into 48 possible permutations, generating a spectrum of tumor biology across 740,246 patients for whom these data were available. The rates of Stage IV disease were calculated across the permutation groups, which were then ranked in order of increasing Stage IV percentage. We aimed to cluster these groups into three subtypes of increasing biological aggressiveness, with up to 25% at the extremes, and the remainder intermediately aggressive. After testing multiple Stage IV percentage cut points in sensitivity analyses, we ultimately classified 22.3% of patients as “indolent,” 61.7% of patients as “intermediate,” and 16.0% of patients as “aggressive.” This process of classification is summarized in Table 1.

**Table 1.** Classification system of breast cancer biological subtypes. ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor 2, LVI: lymphovascular invasion.

Group	n	ER	PR	HER2	Grade	LVI	Row % with Stage IV	N Subtype	Subtype
1	119	-	+	-	1	-	0	165,150	Indolent
2	7	-	+	-	1	+	0		
3	3	-	+	+	1	+	0		
4	146,900	+	+	-	1	-	0.006		
5	12,409	+	-	-	1	-	0.01		
6	3917	+	+	+	1	-	0.012		
7	1795	-	-	-	1	-	0.013		
8	204,447	+	+	-	2	-	0.014	456,894	Intermediate
9	726	+	-	+	1	-	0.018		
10	691	-	+	-	2	-	0.019		
11	21,860	+	-	-	2	-	0.022		
12	12,597	-	-	-	2	-	0.022		
13	52,160	-	-	-	3	-	0.022		
14	9908	+	+	-	1	+	0.022		
15	18,147	+	+	+	2	-	0.026		
16	48,280	+	+	-	3	-	0.026		
17	3123	-	+	-	3	-	0.026		
18	373	-	-	+	1	-	0.027		
19	754	+	-	-	1	+	0.027		
20	14,045	+	-	-	3	-	0.029		
21	98	+	-	+	1	+	0.031		
22	49,161	+	+	-	2	+	0.031		
23	15,296	+	+	+	3	-	0.033		
24	5228	+	-	+	2	-	0.034		



Table 1. Cont.

Group	n	ER	PR	HER2	Grade	LV1	Row % with Stage IV	N Subtype	Subtype
25	5224	–	–	+	2	–	0.038		
26	14,608	–	–	+	3	–	0.038		
27	6336	+	–	+	3	–	0.042		
28	23	–	+	+	1	–	0.043		
29	949	–	+	+	3	–	0.044		
30	2805	–	–	–	2	+	0.045		
31	411	+	+	+	1	+	0.046		
32	5502	+	+	+	2	+	0.046		
33	318	–	+	+	2	–	0.047		
34	4922	+	–	–	2	+	0.048		
35	28,757	+	+	–	3	+	0.051	118,202	Aggressive
36	17,799	–	–	–	3	+	0.056		
37	6178	+	–	–	3	+	0.059		
38	8808	+	+	+	3	+	0.059		
39	1031	–	+	–	3	+	0.065		
40	147	–	–	–	1	+	0.068		
41	1468	+	–	+	2	+	0.068		
42	473	–	+	+	3	+	0.068		
43	175	–	+	–	2	+	0.069		
44	3168	+	–	+	3	+	0.083		
45	7249	–	–	+	3	+	0.086		
46	1665	–	–	+	2	+	0.091		
47	72	–	–	+	1	+	0.097		
48	114	–	+	+	2	+	0.114		

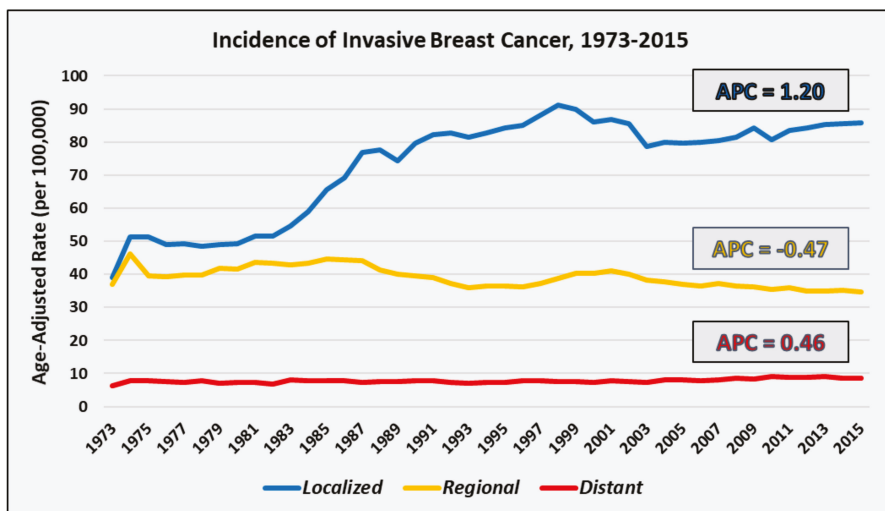
Frequencies of the above biological subtypes were calculated for all the staged breast cancer cases with known biological data, and their distributions were compared across Stages I–IV. Stage I was divided into tumors measuring 0.1–1.0 cm and 1.1–2.0 cm, in order to compare the biology of tiny tumors almost exclusively found on screening mammography with Stage IV biology. Patients without known tumor size or other demographic or disease variables were excluded from the analysis, resulting in 718,118 patients included. Chi-squared testing was used to detect differences in tumor biology across Stages I–IV, with statistical significance set at  $p < 0.05$ .

Finally, multivariable logistic regression using backward elimination tested Stage IV cases for significant associations with the demographic variables, including race/ethnicity, age, insurance, and median household income, as well as with disease variables, including histology, size, and biological category. Only the patients with known demographic and disease variables were included, again totaling 718,118 patients. Type I error was set at  $p = 0.05$ . The analyses were performed using SPSS Statistics software (IBM Version 25, Armonk, NY, USA).

### 3. Results

#### 3.1. Breast Cancer Incidence Trends, 1973–2015

Age-adjusted population-based incidence trends for localized, regional, and distant disease are depicted in Figure 1. Based on SEER 9 registry data, the overall incidence of invasive breast cancer increased between 1973 and 2015. The localized disease rate per 100,000 persons increased from 39.0 in 1973 to 85.9 in 2015, generating an APC of 1.20 (95% CI 0.87–1.53). The regional disease rate per 100,000 slightly decreased from 36.9 in 1973 to 34.7 in 2015, with a negative APC of  $-0.47$  (95% CI  $-0.61$  to  $-0.34$ ). The distant disease rate per 100,000 was lowest but experienced an overall increase from 6.2 in 1973 to 8.7 in 2015, with an APC of 0.46 (95% CI 0.32–0.60).



**Figure 1.** Age-adjusted incidence of localized, regional, and distant breast cancer in women, from 1973 to 2015 (SEER 9). APC: annual percent change.

### 3.2. Demographic and Disease Characteristics of Stage IV Patients

Between 2010 and 2015, there were 992,687 women in the NCDB with staged invasive breast cancer. Of these, 939,903 (94.7%) were Stages I–III, and 52,784 (5.3%) were Stage IV. Univariable analysis of the demographic and tumor characteristics is depicted in Table 2. Analysis of demographic data showed that women with Stage IV disease were more likely to be black, younger than 40 years of age, uninsured or on Medicaid, and living in zip codes where median household income was <\$48,000, and where  $\geq 13\%$  of adult residents did not have a high school degree. Strikingly, Stage IV disease affected 8.0% of blacks, 8.8% of women under 30 years of age, and 14.2% of uninsured. Analysis of tumor data showed that women with Stage IV were more likely to have larger tumors, non-ductal or lobular undifferentiated histologies (“Other”), negative ER or PR status, positive Her2 status, LVI, and poorly differentiated grade. Conspicuously, Stage IV affected 19.0% of tumors > 5cm and 10.9% of tumors with undifferentiated histologies.

### 3.3. Tumor Biology Distribution by Stage

As mentioned in Methods, 740,264 patients had known tumor biology characteristics, including hormone receptor and Her2 status, LVI status, and grade. Of this cohort, 718,118 of patients had known tumor size and other demographic and disease characteristics, without missing data. These patients were included in the analysis of the tumor biological subtypes by stage.

Using the tumor biology classification shown in Table 1, a majority of the cohort, 61.7%, had intermediate biology, while 22.7% had indolent biology and 15.6% had aggressive biology. The distribution of biological categories varied tremendously by disease stage ( $p < 0.001$  for all stages), as shown in Figure 2. Patients presenting with Stages III and IV disease had similar tumor biology and had over eight times the fraction of aggressive tumors and one-seventh the fraction of indolent tumors as patients with small Stage I tumors  $\leq 1$  cm.

**Table 2.** Univariable analysis of demographic and disease variables for American Joint Committee on Cancer Stages I–III and Stage IV. HS: high school.

Demographic/Disease Variables	Stages I–III N (Row%)	Stage IV N (Row%)	p-Value
<b>Race/Ethnicity</b>			
White	738,416 (95.1%)	38,453 (4.9%)	<0.001
Black	103,646 (92.0%)	8988 (8.0%)	
Asian	31,843 (95.6%)	1455 (4.4%)	
Hispanic	50,958 (94.5%)	2983 (5.5%)	
Missing	15,040 (94.3%)	905 (5.7%)	
<b>Age</b>			
<30	4594 (91.2%)	446 (8.8%)	<0.001
30–39	36,589 (93.5%)	2560 (6.5%)	
40–49	141,714 (95.6%)	6553 (4.4%)	
50–59	223,898 (94.6%)	12,803 (5.4%)	
60–69	259,993 (94.8%)	14,212 (5.2%)	
≥70	273,115 (94.4%)	16,210 (5.6%)	
<b>Insurance</b>			
None	17,883 (85.8%)	2961 (14.2%)	<0.001
Private	467,639 (95.9%)	20,062 (4.1%)	
Medicaid	59,468 (90.2%)	6476 (9.8%)	
Medicare	368,966 (94.5%)	21,609 (5.5%)	
Other Government	9705 (95.9%)	411 (4.1%)	
Unknown	16,242 (92.8%)	1265 (7.2%)	
<b>Median Household Income</b>			
≤\$38,000	136,807 (93.2%)	9982 (6.8%)	<0.001
\$38,000–\$47,999	197,306 (94.3%)	11,842 (5.7%)	
\$48,000–\$62,999	251,060 (94.7%)	13,934 (5.3%)	
≥\$63,000	351,943 (95.5%)	16,758 (4.5%)	
Missing	2787 (91.2%)	268 (8.8%)	
<b>Median Education (No HS Diploma)</b>			
≥21%	136,597 (93.3%)	9811 (6.7%)	<0.001
13–20.9%	221,484 (94.1%)	13,914 (5.9%)	
7–12.9%	309,839 (94.9%)	16,810 (5.1%)	
<7%	269,536 (95.7%)	12,002 (4.3%)	
Missing	2447 (90.8%)	247 (9.2%)	
<b>Size (cm)</b>			
0.1–2.0	606,385 (98.7%)	8052 (1.3%)	<0.001
2.1–5.0	268,290 (93.5%)	18,615 (6.5%)	
>5.0	56,294 (81.0%)	13,216 (19.0%)	
Missing	8934 (40.9%)	12,901 (59.1%)	
<b>Histology</b>			
Ductal	704,671 (95.5%)	33,525 (4.5%)	<0.001
Lobular	90,608 (94.2%)	5623 (5.8%)	
Mixed Ductal/ Lobular	49,264 (96.3%)	1912 (3.7%)	
Other	95,360 (89.1%)	11,724 (10.9%)	

Table 2. Cont.

Demographic/Disease Variables	Stages I–III N (Row%)	Stage IV N (Row%)	p-Value
<b>ER</b>			
Negative	155,062 (92.9%)	11,810 (7.1%)	<0.001
Positive	771,633 (95.5%)	36,720 (4.5%)	
Missing	13,208 (75.6%)	4254 (24.4%)	
<b>PR</b>			
Negative	243,415 (93.0%)	18,354 (7.0%)	<0.001
Positive	681,482 (95.8%)	29,586 (4.2%)	
Missing	15,006 (75.6%)	4844 (24.4%)	
<b>Her2</b>			
Negative	763,436 (91.5%)	33,677 (4.2%)	<0.001
Positive	119,352 (91.5%)	11,146 (8.5%)	
Missing	57,115 (87.8%)	7961 (12.2%)	
<b>Grade</b>			
Well-Differentiated	212,301 (98.6%)	3107 (1.4%)	<0.001
Moderately Differentiated	398,667 (96.1%)	16,333 (3.9%)	
Poorly Differentiated	270,103 (93.4%)	18,953 (6.6%)	
Missing	58,832 (80.3%)	14,391 (19.7%)	
<b>LVI</b>			
Negative	628,236 (98.1%)	12,155 (1.9%)	<0.001
Positive	154,951 (95.0%)	8237 (5.0%)	
Missing	156,716 (82.9%)	32,392 (17.1%)	

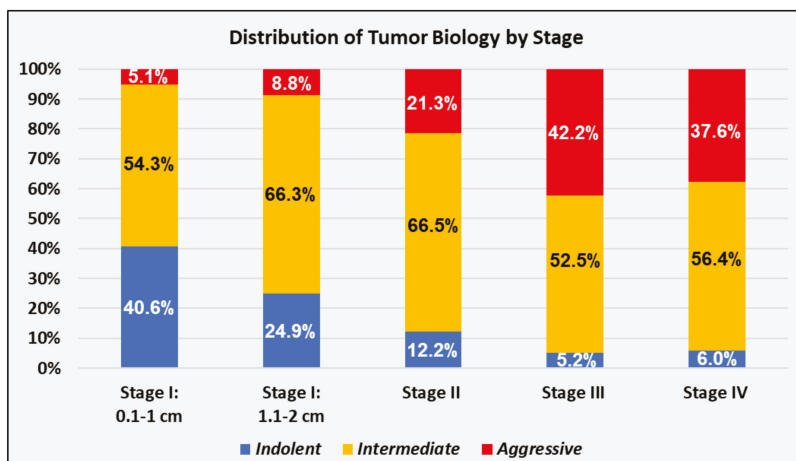


Figure 2. Tumor biology by AJCC 7th edition stage.

### 3.4. Multivariable Analysis of Demographic and Disease Characteristics

In the multivariable logistic regression model, many demographic and disease variables that were associated with Stage IV disease in univariable analysis remained significantly associated, as shown in Table 3. Large tumor size, aggressive biological subtype, and no insurance were the strongest predictors. Tumors with size >5 cm were more than 15 times as likely to predict Stage IV disease as tumors with size ≤2 cm (OR 15.6, 95% CI 14.9–16.5), and tumors with aggressive biology were more than 3 times as likely to present with Stage IV disease as indolent tumors (OR 3.22, 95% CI 2.99–3.47).

Race/ethnicity, age, household income, and histology remained significant in the model, but their effects were fairly minor.

**Table 3.** Multivariable logistic regression demonstrating the strength of association between Stage IV disease and demographic and disease factors.

Demographic/Disease Factors	Stage IV (De Novo) Odds Ratio	95% Confidence Interval
<b>Race/Ethnicity</b>		
White	Reference	Reference
Black	1.09	1.04–1.15
Asian	0.78	0.71–0.86
Hispanic	0.71	0.66–0.77
<b>Age</b>		
<30	1.38	1.17–1.63
30–39	1.08	0.99–1.18
40–49	0.87	0.81–0.93
50–59	1.07	1.01–1.14
60–69	1.06	1.01–1.12
≥70	Reference	Reference
<b>Insurance</b>		
None	Reference	Reference
Private	0.43	0.39–0.46
Medicaid	0.74	0.67–0.81
Medicare	0.51	0.47–0.56
Other Government	0.41	0.34–0.50
Unknown	0.57	0.49–0.68
<b>Median Household Income</b>		
≤\$38,000	1.12	1.06–1.18
\$38,000–\$47,999	1.09	1.04–1.14
\$48,000–\$62,999	1.04	0.99–1.08
≥\$63,000	Reference	Reference
<b>Size (cm)</b>		
0.1–2.0	Reference	Reference
2.1–5.0	4.40	4.21–4.60
>5.0	15.6	14.9–16.5
<b>Histology</b>		
Ductal	Reference	Reference
Lobular	0.83	0.78–0.88
Mixed Ductal/Lobular	0.82	0.75–0.88
Other	0.84	0.79–0.89
<b>Biological Subtype</b>		
Indolent	Reference	Reference
Intermediate	2.05	1.91–2.20
Aggressive	3.22	2.99–3.47

#### 4. Discussion

Multiple studies have shown that, despite widespread screening programs in the United States, the incidence of Stage IV breast cancer has remained stable or increased over time [1–3,5,6]. In this study, we analyzed the most recent and comprehensive population-based SEER data and showed that the incidence of Stage IV disease has indeed been gradually increasing in recent decades.

Esserman and colleagues elegantly delineated this issue in 2009, contrasting the expected stage-based incidence of a theoretically effective disease-screening program with the actual breast cancer incidence trends since the rise of screening in the early 1980s. They highlighted that, while successful screening programs are able to downstage incident cancers over time, breast cancer screening has generated more localized disease diagnoses without congruently diminishing the incidence of advanced cancer [1].

Welch and colleagues pointed out that since the advent of widespread mammography screening, small cancers under 2 cm have increased in incidence over three times more than large tumors over 2 cm have decreased [7]. The clear implication is that not all small cancers are destined to become large cancers, and this leads to overdiagnosis. Lannin and Wang provided an explanation for this by comparing the biology of small and large cancers [4,8]. They found that many breast cancers are small, not because they are detected early, but because they have favorable biology. The current study is an extension of that work and shows that cancers presenting with distant metastases are a distinct subpopulation with a biology much more aggressive than that of the small tumors found by screening mammography. Only a small fraction of the tumors found by screening mammography have the biological profile that puts them at risk for *de novo* Stage IV disease.

Of course, there are other possible explanations for increasing Stage IV incidence. One contributing factor could be stage migration, the phenomenon whereby the use of high-resolution imaging, including positron emission/computed tomography scans and magnetic resonance imaging, leads to more frequent discovery of distant disease [9–11]. It seems unlikely, however, that this would precisely counterbalance a decline in advanced cancer diagnoses that might otherwise be seen from early mammographic detection.

Our data complement the body of literature suggesting that Stage IV cancers arise in unscreened and underprivileged populations, including the very young, the very old, and the disadvantaged with respect to healthcare access and quality. In our analyses, women younger than 40 years and older than 70 years of age had higher rates of Stage IV disease when compared to women aged 40–69 years, who are known to have the highest rates of disease screening [12]. Black women, known to suffer disparate breast cancer outcomes, had significantly higher rates of Stage IV disease than white women [13,14]. Those without health insurance and with Medicaid, as well as from regions in the lowest brackets for income and education—all of which imply low resource settings—also disproportionately presented with Stage IV disease [15–17]. These statistics must be interpreted cautiously, however, as NCDB data are not population-based and thus do not differentiate between higher incidence of Stage IV disease versus relatively lower incidence of Stages I–III disease. In fact, Welch and colleagues used population-based SEER data to show that poorer counties with less mammographic screening have lower overall breast cancer incidence but similar Stage IV disease incidence and cancer mortality compared with wealthier counties [18,19].

Even when adjusting for demographic and socioeconomic factors, our data support the concept that Stage IV tumors represent a unique subpopulation and are biologically distinct from the small, indolent tumors usually detected by mammography. Other studies have postulated that breast cancer presenting at an advanced stage may be innately endowed with biologic machinery that promotes swift growth and spread during the 12–24 months interval between mammograms [1,20,21]. Our findings give credence to this theory by highlighting the uniquely aggressive features of Stage IV disease.

In a previous study, Lannin used ER, PR, and grade to stratify patients into three prognostic groups based on breast cancer-specific survival, terming the groups “favorable”, “intermediate”, and “unfavorable”. In this study, we added two additional variables to the model—Her2 and LVI—and

used de novo Stage IV disease as the target outcome. These five tumor characteristics were specifically chosen to reflect the biological behavior that predicts distant metastases, thus the group names were changed to “aggressive”, “intermediate”, and “indolent”.

Her2 is a well-known marker for biological aggressiveness and was formerly associated with poor prognosis. With effective targeted therapy, it currently confers a better than average prognosis and yet it is strongly associated with Stage IV disease [22–26]. LVI similarly marks aggressive growth patterns [27,28]. Unfortunately, its status was missing in nearly 20% of the dataset and well over half of Stage IV cases. This may be explained by the fact that many patients with Stage IV disease undergo needle biopsy only, and pathologists are either unable or unmotivated to evaluate for LVI. Despite missing data, we included LVI in our model, as it was highly informative of biology when known. As shown in Table 1, 18 of 19 groups with the highest rate of Stage IV disease presentation were positive for LVI. All 24 groups included in the “aggressive” category were positive for either LVI or Her2. The most biologically unfavorable cancers from the earlier prognostic-based model—triple negative with grades 2 and 3—only cluster to the current “aggressive” category when positive for LVI.

Tumor biology is evolving to become a critical factor in estimating prognosis and guiding treatment. In the AJCC 8th edition disease staging system, anatomical features like tumor size and nodal status are considered insufficient to accurately inform the stage and treatment plan, particularly in the developed world where testing for biomarkers is ubiquitous. The variables used in this study are primitive measures of tumor biology compared to molecular and genomic assays such as OncotypeDx or MammaPrint [4,29–31]. However, they are readily available in large datasets like SEER and the NCDB for use in estimating population trends in tumor biology. Future studies will likely elucidate more sophisticated biological mechanisms responsible for aggressive Stage IV tumors. It seems likely that these differing biological characteristics will explain why the incidence of Stage IV breast cancer has not decreased with screening mammography.

## 5. Conclusions

The incidence of de novo Stage IV breast cancer is increasing in the United States despite widespread mammography screening. This is likely related to the differing populations of biologic tumor types that comprise Stage IV tumors versus early-stage tumors commonly found on mammography. Our analysis demonstrates that aggressive tumor biology accounts for nearly 40% of advanced-stage tumors, versus only 5% of tiny early-stage tumors. Conversely, indolent biology is rarely associated with advanced disease. Aggressive biology resulting in insidious growth patterns may explain why the incidence of Stage IV cancer has not decreased with screening.

**Author Contributions:** Conceptualization, methodology, data curation, formal analysis, and preparation of the original draft of this study were performed by D.R.H. and D.R.L. A.S.C., K.F., and B.K.K. contributed to the data curation, formal analysis, and review and editing of the manuscript. B.K.K. and D.R.L. also supervised the study.

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## Abbreviations

SEER	Surveillance, Epidemiology, and End Results
NCDB	National Cancer Data Base
AJCC	American Joint Committee on Cancer
APC	Annual Percent Change
CI	Confidence Interval
ER	Estrogen Receptor
PR	Progesterone Receptor
Her2	Human Epidermal Growth Factor 2
LVI	Lymphovascular Invasion
OR	Odds Ratio

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Article

# Impact of Overdiagnosis on Long-Term Breast Cancer Survival

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**Abstract:** Elucidating whether and how long-term survival of breast cancer is mainly due to cure after early detection and effective treatment and therapy or overdiagnosis resulting from the widespread use of mammography provides a new insight into the role mammography plays in screening, surveillance, and treatment of breast cancer. Given information on detection modes, the impact of overdiagnosis due to mammography screening on long-term breast cancer survival was quantitatively assessed by applying a zero (cured or overdiagnosis)-inflated model design and analysis to a 15-year follow-up breast cancer cohort in Dalarna, Sweden. The probability for non-progressive breast cancer (the zero part) was 56.14% including the 44.34% complete cure after early detection and initial treatment and a small 11.80% overdiagnosis resulting from mammography screening program (8.94%) and high awareness (2.86%). The 15-year adjusted cumulative survival of breast cancer was dropped from 88.25% to 74.80% after correcting for the zero-inflated part of overdiagnosis. The present findings reveal that the majority of survivors among women diagnosed with breast cancer could be attributed to the cure resulting from mammography screening and accompanying effective treatment and therapy and only a small fraction of those were due to overdiagnosis.

**Keywords:** overdiagnosis; mammography screening; invasive breast cancer; zero-inflated Poisson regression model

## 1. Introduction

While the prognosis of breast cancer (BC) has been substantially improved due to early detection of breast cancer attributed to the widespread use of mammography, the issue of overdiagnosis resulting from mammography screening has been debated over the past decade [1–5]. As these overdiagnosed

cases are biologically indolent and non-progressive they would have never progressed to clinical phase and caused death due to breast cancer during the patients' lifetime, implying that any treatment was unnecessary and would not have been administered had screening not been applied to these women [6–10].

The previous studies on the extent of overdiagnosis were estimated by excess incidence due to screening compared with background incidence derived from randomized control trials or predicted incidence extrapolated from previous unexposed epochs, making allowance for lead-time [2,9,11,12]. Note that these previous methods, while estimating the proportion of overdiagnosis, require individual normal and incident breast cancer data and also a strong assumption of lead-time distribution. These traditional approaches cannot be used for assessing the impact of overdiagnosis on long-term survival when only information on breast cancer cases and deaths from breast cancer is available.

Here, we propose a new approach to estimating overdiagnosis using information on the survival of breast cancer detected by different modalities (detection modes) together with prognostic factors with the premise that overdiagnosis of BC would not result in deaths from breast cancer. However, the survivors of these overdiagnosed BCs are often indistinguishable from those with non-overdiagnosed BC cases but without potential of dying from breast cancer due to effective initial treatment and therapy, namely the completely cured. Both types are regarded as non-progressive BC with zero-probability of dying from BC but have manifestly different causes. To distinguish the completely cured patients from overdiagnosed ones requires information on detection mode such as screen-detected cases, interval cancers, and cancers in non-participants. The overestimation of cumulative survival due to the zero-probability of dying from breast cancer resulting from overdiagnosis would be expected if these overdiagnosed cases cannot not be separated from the completely cured.

Moreover, the non-progressive BCs indicated above would also be mixed up with progressive BC patients still alive at each specific follow-up timepoint. Whether and when these progressive cases would die from BC is highly dependent on subsequent treatments and therapies and prognostic factors [13–18]. However, only relying on these prognostics may not be sufficient to distinguish between progressive and non-progressive BC because excellent survival tumors with good prognostic factors may also be a consequence of overdiagnosis due to mammography [19].

The aim of this study is therefore to apply the zero-inflated regression model to estimate the proportion of overdiagnosis resulting from mammography screening separated from the proportion of the completely cured due to effective treatment and therapy. We also assess the cumulative survival after correction for the zero-inflated part of overdiagnosis.

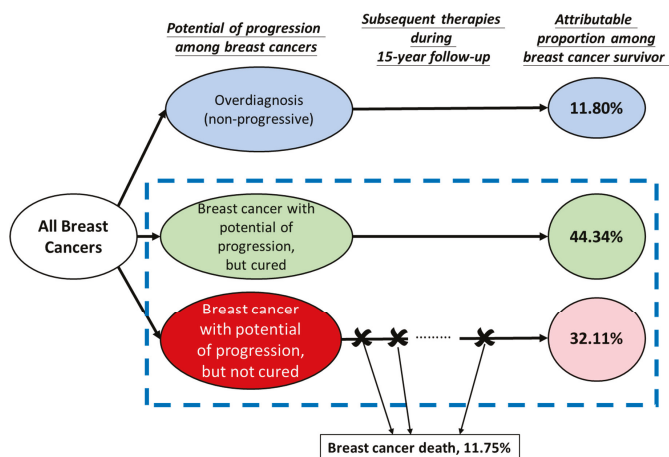
## 2. Materials and Methods

### 2.1. Study Subjects and Design

We quantified the respective contributions of overdiagnosis attributable to mammography and cures due to early detection and effective treatment by using a cohort composed of 1346 patients diagnosed with invasive BC at Falun Central Hospital of Dalarna County in Sweden in two periods with available information on prognostic factors, from 1996 to 1998 and from 2006 to 2010, in combination with a zero-inflated model design and analysis. The main reason of selecting two periods is mainly due to available information on immunohistochemical (IHC) markers, particularly HER2, which had not been widely tested before 2005. The period of 1996–1998 was a pilot phase for collecting such information. The two cohorts were followed over time until the end of 2010. Note that breast cancer service screening program with mammography has been offered since 1985 at the close of the Swedish Two-county randomized controlled trial [20].

In addition to longitudinal follow-up data, the current study design illustrated in Figure 1 is based on the concept of the zero-inflated model for solving the problem of being unable to distinguish between overdiagnosed cases from cured cases due to effective treatment and therapies as mentioned in

the introduction. All diagnosed breast cancers are classified into three types according to the potential for progression and the cure after initial treatment. The top left circle represents overdiagnosed cases (blue) with zero probability of dying from breast cancer mainly resulting from mammography screening. The dotted box is composed of those breast cancers with potential of progression, which are further divided into two types, the cured after initial treatment (green) and the cured after subsequent therapies during 15-year follow-up (red). The final column is the estimated attributable proportions among three types of survivors of breast cancer. If there is a lack of information on detection mode it is very difficult to distinguish the cured from the overdiagnosed. The screened cohort together with the collection of these prognostic factors provide an opportunity to distinguish overdiagnosis from the cured. The derivation of percentages among breast cancer cases delineated in Figure 1 is elaborated in the Statistical Analysis section and Appendix



**Figure 1.** Study design for estimating the proportion of breast cancer survivors attributed to overdiagnosis, the completely cured after initial treatment, and the curation after subsequent therapies during 15-year follow-up.

## 2.2. Detection Mode Related to Curation and Overdiagnosis

There are three detection modes, screen-detected cases, interval cancers, and cancers from non-participants or outside the age ranges of screening. Here we assume overdiagnosis of BC due to mammography screening can only result from screen-detected cases as they were detected though mammography. Interval cancers after the exposure to a previous screen with negative findings were detected either through possible self-referral of patients or due to the presence of symptoms and signs. Cancers from non-participants or outside screening were diagnosed due to the presence of symptom and signs. In this sense, interval cancers would enhance awareness of being diagnosed as BC compared to cancers from non-participants. This can be supported by the fact that interval cancers have higher survival than cancers from non-participants [21]. Suppose treatment and therapies were administered to three groups according to the indication for the choice of treatment modality based on significant prognostic factors. The difference of zero probability on death from BC between screen-detected cancers and interval cancers would provide information on excess zeros due to overdiagnosis resulting from mammography. The difference of zero probability between interval cancers and cancers from non-participant offers information on overdiagnosis due to increased awareness. Details of the calculation are given in the statistical section.

### 2.3. Prognostic Factors

We collected factors responsible for progressive BCs including conventional tumor attributes (size, lymph node involvement, and histological grade), three immunohistochemical markers (ER, PR, HER2), triple negative (defined by these three IHC markers), surgical treatment and adjuvant therapy. Conventional tumor attributes have been collected since the dawn of the service screening; Surgical treatment (breast-conserving surgery, mastectomy, or others), and adjuvant therapy (radiotherapy, chemotherapy, or hormone therapy) had been collected since 1996.

Data on tumor phenotypes related to IHC markers including ER, PR and HER2 status were collected retrospectively for the period of 1996 to 1998 by standard antibody staining in the largest invasive tumor component for each patient and was described in full in previous studies [22]. The antibodies (supplier, type, dilution) used for staining are delineated as follows: ER (clone SP1; Ventana Medical Systems, Tucson, AZ, USA; 1:200 dilution), PR (clone PgR 636; Dako, Glostrup, Denmark; 1:50 dilution), and HER2 (code A 0485; Dako; 1:250 dilution). The cut-off point for ER and PR positivity is nuclear staining >10% of tumor cells. The criteria of HER2 positivity was offered by manufacturer. Triple negative BC is defined as a breast tumor with all ER, PR, and HER2 being negative.

### 2.4. Statistical Analysis

Descriptive data are presented with frequency and percentage. For categorical data, the chi-square test was used to compare the difference between groups and the Fisher Exact test was used if any count was less than 5. We first applied the Poisson regression model assuming the number of BC deaths follows a Poisson distribution. We estimated follow-up women years from the date of diagnosis as BC to the date of death from BC, loss of follow-up, or the end of this study as the offset in Poisson regression model. The value of deviance divided by degree of freedom provides an indicator to assess the extent of over-dispersion and under-dispersion for the specified Poisson regression model. For the elucidation of overdiagnosis in BC, we applied the zero-inflated Poisson (ZIP) regression model [23], which is a mixture of a Poisson regression model (count part) and a logistic regression model (zero part) as derived in Appendix A. The former model (Poisson regression model, count part) was used to evaluate the prognostic factors for progressive BCs. The prognostic factors included three conventional tumor attributes and treatment and therapies (surgery, chemotherapy, radiotherapy, hormone therapy). The latter model (logistic regression model, zero part) was used to estimate the probability of zero part (including overdiagnosis cases or cure after initial treatment and therapies) for non-progressive BCs. We used the detection mode of cancers as covariates to distinguish two types in the zero part (Appendix A). We then used the regression coefficients of logistic regression part in the ZIP model to calculate the probability of zero among all BCs and respective probabilities of zero by detection mode as detailed in Appendix A. The probability of overdiagnosis due to mammography screening and enhanced awareness is calculated as follows:

$$\begin{aligned} & \text{The probability of overdiagnosis due to mammography screening} = \\ & ((\text{The probability of zero for screen-detected} - \text{the probability of zero for interval cancer}) \quad (1) \\ & \quad \times \text{The probability of zero among all BCs}) \end{aligned}$$

$$\begin{aligned} & \text{The probability of overdiagnosis due to awareness} \\ & = ((\text{the probability of zero for interval cancers} - \text{the probability of zero for cancers from non-participants}) \quad (2) \\ & \quad \times \text{the probability of zero among all BCs}) \end{aligned}$$

$$\text{The probability of cure due to treatment} = \text{The probability of zero among all BCs} - ((1) + (2)) \quad (3)$$

We further derive 15-year cumulative survival curves with and without correcting for overdiagnosis by using the hazard rate derived from the ZIP model and the corresponding figure from the conventional Poisson regression model without considering overdiagnosis as described in

Appendix A). Two-sided  $p$ -value less than 0.05 was treated as statistical significance. All analyses were conducted with SAS version 9.4 (SAS Institute, Cary, NC, USA).

### 2.5. Ethics Approval

This study was approved by the Joint Institutional Review Board of Taipei Medical University (TMU-JIRB, approval numbers N201607008).

### 3. Results

Table 1 shows the frequencies of age at diagnosis, first generation prognostic factors (tumor size, node status, histologic malignancy grade), IHC markers (ER, PR, HER2, triple negative), and treatment and therapies by BC death. The distribution of age at diagnosis was similar between women who died from BC and those who did not. The distributions of tumor size, status of node involvement, histological grade, ER, PR, triple negative, surgery, and hormonal therapy were significantly different ( $p$ -value < 0.05) according to BC death. Women who had tumor size larger than 20 mm, positive nodes, grade 3, ER(−), PR(−), and triple negative were more likely to die from BC.

**Table 1.** The distribution of age at diagnosis, conventional tumor attributes, IHC markers (ER, PR, HER2, Triple negative), mammographic appearance, and treatment by status of breast cancer death.

Variable/Level	Breast Cancer Death				$p$ -Value
	No ( $n = 1228$ )	%	Yes ( $n = 118$ )	%	
Age at diagnosis					0.345
<50	202	92.7	16	7.3	
50–69	596	91.8	53	8.2	
70+	430	89.8	49	10.2	
Size *, mm					<0.001
1–9	233	98.3	4	1.7	
10–14	273	96.1	11	3.9	
15–19	260	95.2	13	4.8	
20–29	263	87.4	38	12.6	
≥30	155	83.8	30	16.2	
Nodes *					<0.001
Negative	805	95.4	39	4.6	
Positive	390	87.2	57	12.8	
Grade *					<0.001
1	284	97.3	8	2.7	
2	633	93.6	43	6.4	
3	263	85.4	45	14.6	
ER *					<0.001
Negative	174	84.1	33	15.9	
Positive	990	94.6	57	5.4	
PR *					<0.001
Negative	448	87.3	65	12.7	
Positive	714	96.6	25	3.4	
HER2 *					0.8771
Negative	1018	92.9	78	7.1	
Positive	149	92.5	12	7.5	
Triple negative *					<0.0001
Yes	115	81.6	26	18.4	
No	1046	94.2	64	5.8	

Table 1. Cont.

Variable/Level	Breast Cancer Death				p-Value
	No (n = 1228)	%	Yes (n = 118)	%	
Surgery *					<0.0001
MA	452	87.8	63	12.2	
BCS	538	95.9	23	4.1	
Others	238	88.1	32	11.9	
Chemotherapy					0.2018
Yes	270	89.4	32	10.6	
No	958	91.8	86	8.2	
Radiotherapy					0.8979
Yes	632	91.3	60	8.7	
No	596	91.1	58	8.9	
Tamoxifen					0.0061
Yes	480	93.9	31	6.1	
No	748	89.6	87	10.4	

Abbreviations: ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; IHC markers: immunohistochemical markers; PR: progesterone receptor; BCS: breast conserving surgery; MA: mastectomy. \* 66 subjects had no information on tumor size (44 survivors, 22 deaths), 55 subjects had no information on nodal involvement (33 survivors, 22 deaths), 70 subjects had no information on histological grade (48 survivors, 22 deaths), 92 subjects had no information on ER status (64 survivors, 28 deaths), 94 subjects had no information on PR status (66 survivors, 28 deaths), 89 subjects had no information on HER2 status (61 survivors, 28 deaths), 95 subjects had no information on triple negative status (67 survivors, 28 deaths).

Table 2 shows that conventional tumor attributes were significant predictors in both univariate and multivariable models. The crude RR was significantly higher for tumor with size 20–29 mm (9.32; 95% CI, 3.33–26.13) and 30 mm+ (13.65; 95% CI, 4.81–38.74) compared with size 1–9 mm, tumor with node positive (3.70; 95% CI, 2.46–5.57) compared with node negative, tumor with grade 3 (2.97; 95% CI, 1.99–4.43) compared with grade 1/2, and triple negative (3.32; 95% CI, 2.11–5.24) compared with non-triple negative cancers. In the multivariable analysis, tumor with size 20–29 mm (aRR = 2.63; 95% CI, 1.38–5.02) and 30+ mm (aRR = 2.39; 95% CI, 1.19–4.80) were at greater risk than those with size 1–9 mm. Positive node led to an elevated risk (aRR = 1.86; 95% CI, 1.18–2.94) as opposed to negative node after adjusting for variables related to treatment such as surgery, chemotherapy, radiotherapy, and hormonal therapy. Interpretation of effect size on treatment and therapies should be taken with great caution as they are not a reflection of efficacy of treatment and therapies but an indication for treatment and therapies according to tumor attributes. These accounted for the findings that those with mastectomy and radiotherapy had higher hazard of dying from breast cancer and insignificant effective chemotherapies and tamoxifen therapy even after adjustment for other significant prognostic factors.

The value of deviance divided by the degree of freedom, an indicator for assessing the level of over-dispersion, was about 0.46–0.59 in the univariate model and 0.49 in the multivariable model. As this value was less than 1, it strongly suggests the problem of under-dispersion (excess zeros).

We used data with complete information ( $n = 1233$ ) on conventional tumor attributes, variables related to surgery and adjuvant therapy, and detection mode of BCs for the ZIP model analysis. The larger the value of odds ratio (OR), the higher probability to be cured after initial treatment or overdiagnosis. The larger the value of relative risk (RR), the higher the risk of dying from BC. Table 3 shows the estimated parameters, ORs, and RRs for the ZIP model.

Tumor size, node status, grade were significant factors related to risk of dying from BC after considering treatment. Compared with non-participants and outside screening of BCs, screen detected cancers and interval cancers were with higher odds (OR = 2.38, 95% CI: 0.97–5.85 and OR = 1.23, 95% CI: 0.48–3.17, respectively) of being zero.

**Table 2.** The univariate and multivariable analysis of Poisson regression model for predicting breast cancer death by conventional tumor attributes and other predictors.

Variable/Level	Univariate			Multivariable		
	cRR (95% CI)	p-Value	Deviance/df.	aRR (95% CI)	p-Value	Deviance/df.
Tumor size, mm		<0.001	0.46		<0.001	
10–14 vs. 1–9	2.53 (0.80–7.93)			1.01 (0.45–2.24)		
15–19 vs. 1–9	3.12 (1.02–9.56)			1.12 (0.52–2.43)		
20–29 vs. 1–9	9.32 (3.33–26.13)			2.63 (1.38–5.02)		
30+ vs. 1–9	13.65 (4.81–38.74)			2.39 (1.19–4.80)		
Node (+) vs. (–)	3.70 (2.46–5.57)	<0.001	0.46	1.86 (1.18–2.94)	0.007	
Grade 3 vs. 1/2	2.97 (1.99–4.43)	<0.001	0.48	1.32 (0.84–2.07)	0.228	
Triple negative						0.49
Yes vs. No	3.32 (2.11–5.24)	<0.001	0.47	1.53 (0.89–2.63)	0.132	
Surgery MA vs. BCS	4.02 (2.49–6.48)	<0.001	0.55	2.79 (1.56–4.98)	<0.001	
Chemotherapy						
Yes vs. no	1.58 (1.05–2.37)	0.027	0.59	0.83 (0.51–1.38)	0.474	
Radiotherapy Yes vs. no	0.71 (0.50–1.02)	0.063	0.59	1.39 (0.82–2.37)	0.215	
Tamoxifen Yes vs. no	0.96 (0.64–1.45)	0.849	0.59	0.89 (0.56–1.42)	0.633	

Abbreviations: aRR: adjusted relative risk; cRR: crude relative risk; df.: degree of freedom; MA: Mastectomy; BCS: Breast-conserving surgery.

The probability of zero part among all non-progressive BC was 56.14%. The corresponding probabilities for screen detected cancer, interval cancer, and refuser/outside screening cancers were 66.42%, 50.50%, and 45.40% respectively, which gave 8.94% overdiagnosis due to mammography screening and 2.86% due to high awareness for those interval cancers but exposed to mammography screening based on the equation (1) and (2). The probability of zero due to the curation resulting from early detection and effective treatment was 44.34% (Figure 1, green).

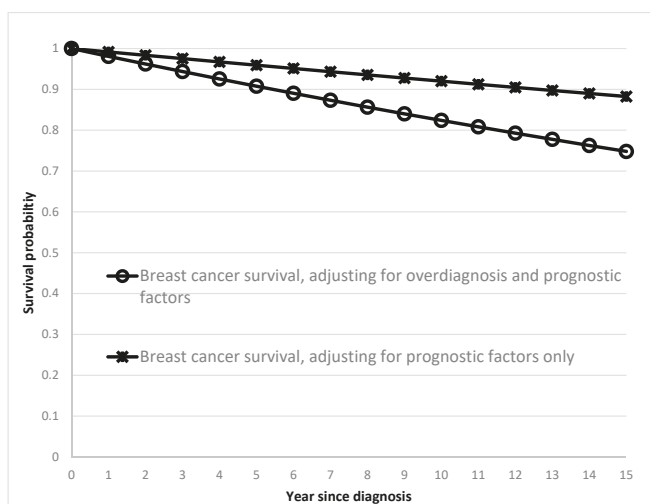
The 15-year prognosis-adjusted cumulative survival of BC after correcting for overdiagnosis fell from 88.25% (Figure 2, cross mark) to 74.80% (Figure 2, hollow circle) after further adjustment for prognostic factors in the count part of progressive BC (Figure 1, red). The 15-year survival rate among 43.86% progressive BC after subsequent treatments and adjuvant therapies was 32.11% after adjustment for significant prognostic factors (Figure 1, pink).

**Table 3.** The regression coefficient of Zero-inflated Poisson regression model and overdiagnosis rate.

Variable	Regression Coefficient	S.E.	RR/OR (95% CI)	p-Value
<b>Count Part</b>			<b>RR</b>	
Intercept	−6.216	0.830		
Size, mm				0.015
10–14 vs. 1–9	1.307	0.808	3.69 (0.76–18.01)	
15–19 vs. 1–9	1.348	0.802	3.85 (0.80–18.53)	
20–29 vs. 1–9	2.329	0.769	10.26 (2.27–46.33)	
30+ vs. 1–9	2.246	0.791	9.45 (2.01–44.49)	
Node (+) vs. (–)	0.877	0.315	2.40 (1.30–4.45)	0.005
Grade 3 vs. 1/2	0.484	0.276	1.62 (0.94–2.79)	0.080
Surgery MA vs. BCS	0.651	0.360	1.92 (0.95–3.88)	0.071
Triple Negative Yes vs. No	0.914	0.311	2.49 (1.36–4.59)	0.003
Chemotherapy Yes vs. No	−0.238	0.319	0.79 (0.42–1.47)	0.456
Radiotherapy Yes vs. No	0.210	0.367	1.23 (0.60–2.53)	0.568
Tamoxifen Yes vs. No	−0.054	0.281	0.95 (0.94–1.64)	0.847
<b>Zero Part</b>			<b>OR</b>	
Intercept	−0.185	0.381		
Detection mode				0.041
SD vs. RF	0.867	0.459	2.38(0.97–5.85)	
IC vs. RF	0.205	0.484	1.23(0.48–3.17)	

Abbreviations: S.E.: Standard error; MA: Mastectomy; BCS: Breast-conserving surgery; SD: screen detected cancer; IC: interval cancer; RF: refuser & outside screening cancers.





**Figure 2.** Cumulative survival of breast cancer-based models with and without considering overdiagnosis.

#### 4. Discussion

The long-term prognosis of BC has been substantially improved over the past three decades due to early detection, mainly through mammographic screening. However, the harm of overdiagnosis is a concomitant risk of the benefit of mammography screening and it has now become a debatable issue and concern for population-based mammography screening over the past decade [1–5]. For breast cancer cases with overdiagnosis, there is 0% probability of dying from BC and treatment is unnecessary for them. It may also result in the overestimation of cumulative survival attributed to effective treatment and therapies in accompany with early detection of mammography screening. The survival of BC would thus be artificially inflated if such zero-inflated overdiagnosis is included. Estimating the quantity of overdiagnosis separated from the cured due to treatment is intractable but indispensable and can be truly a reflection of early detection and effective treatment and therapy. Our novel approach with the zero-inflated design and model for separating the cured from the overdiagnosed provides a solution but the conventional statistical model could not distinguish the completed cured after initial treatment (green, Figure 1) and the curation after subsequent therapies during 15-year follow-up (red, Figure 1). From the viewpoint of methodology, the use of the zero-inflated model enables us to separate the zero part with potential of progression but completely cured after initial treatment from the non-zero part with potential of progression but cured after subsequent therapies during 15-year follow-up particularly when tumor attributes related to breast cancer progression were considered in the non-zero (progressive) part.

In addition to the assessment of the impact of overdiagnosis on long-term survival, our proposed zero-inflated model also provides an insight into the proportion of overdiagnosis resulting from mammography screening that has been well studied in previous studies using excess incidence approach with lead-time adjustment [2,9,11,12]. After reviewing the primary articles that estimated the overdiagnosis level in European population-based mammography screening programs, Puliti et al. found that the rates of overdiagnosis of invasive BCs due to mammography screening varied from 0 to 54% [11]. Morrel et al. reported lower estimated baseline incidence resulted in higher level of overdiagnosis (42% vs. 30%) [24]. They also reported that longer lead-time (5 years vs. 2.5 years) contributed to lower extent of overdiagnosis (42% vs. 51%) [24]. Different background incidence rates and the assumption of lead-time distribution may account for such a wide range of estimates on overdiagnosis reported before. Several studies reported that the overdiagnosis rate was different by

age [25,26]. In addition to the disparity in the methodology of lead-time adjustment and the extent of mammography screening, variation of overdiagnosis across age may also be explained by the fact that background incidence rate and the distribution of lead-time also vary with age [2,9,11,12].

Our proposed alternative approach to evaluating the extent of overdiagnosis dispenses with background incidence of BC and the assumption of lead-time distribution. We only used empirical data on BCs with available information on detection mode, treatment and therapies, and prognostic factors collected from an organized service screening program after population-based randomized controlled trial on mammography screening since 1977 in Falun (also known as Dalarna now, and Kopparberg in the 1990s), Sweden [27]. This empirical data is well suited to estimate the overdiagnosis from mammography screening and enhanced awareness as the attendance rate of mammography screening was over 80% and women in this county were also with high awareness of being diagnosed as BC through interval cancers [4,6]. Information on BCs with various detection modes is therefore useful for separating the completed cured from overdiagnosis.

It is very interesting to note that the probability of being zero part among interval cancers was higher than refuser/outside screening BCs. The difference might result from high awareness of detecting BCs through interval cancers because they had been exposed to mammography screening. Our result showed about 3% overdiagnosis due to enhanced awareness of detecting BC through interval cancers.

There are two limitations of the current study. Although the application of ZIP enables us to estimate the attributable proportions of three types of breast cancer survivors, personalized prediction for three types cannot be achieved without more updated information on molecular and imaging biomarkers can be included in the zero part and non-zero part, respectively. The second is related to the validation of this zero-inflated model by the application of the proposed model together with the estimated parameters to independent prospective follow-up data of this cohort in the future and also to data outside this country. We therefore strongly suggest here that our proposed zero-inflated model had better be applied to other countries in Europe where mammography screening programs have been widely served since the 1990s and the screening rate was also high in order to see whether and how the cure, overdiagnosis, and the survival of progressive BCs vary with different service screening programs. We also suggest that our model can be applied to regions with lower mammography screening rates and lower awareness of detecting BC in contrast to the current data with high careening rate and enhanced awareness in order to test the generalizability of our proposed zero-inflated regression.

## 5. Conclusions

In conclusion, the zero-inflated model design is a novel approach to correcting cumulative survival of early-detected BC inflated due to the zero part of overdiagnosis. Application of this model to the Dalarna breast cancer service screening program revealed that, among all breast cancers detected from this program, there were 76% survivors (44% completely cured and 32% still alive) due to early detection of mammography and effective treatment after 15 years of follow-up and overdiagnosis accounted for 12% of survivors.

**Author Contributions:** Study concept and design: H.-H.C.; Acquisition and interpretation of data: W.-H.K., C.-P.Y., K.-J.C., L.T.; Literature search: K.-J.C., W.-H.K., C.-P.Y., J.C.-Y.F.; Drafting of the manuscript: J.C.-Y.F., C.-Y.H., K.-J.C., H.-H.C.; Critical revision of the manuscript for important intellectual content: L.T., K.-J.C., H.-H.C.; Statistical analysis: C.-Y.H., J.C.-Y.F., S.L.-S.C., A.M.-F.Y.; Obtained funding: S.L.-S.C., A.M.-F.Y., H.-H.C.; Administrative, technical, or material support: A.M.-F.Y., S.L.-S.C.

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**Conflicts of Interest:** The authors declare no conflict of interest.

### Appendix A Zero-Inflated Poisson Regression Model

Let  $Y$  denote the random variable representing the observed counts of breast cancer death. The effect of demographic factors, tumor attributes, IHC (immunohistochemical markers), type of treatments and therapies for progressive BCs can be evaluated by using Poisson regression model specified as follows. The number of breast cancer death ( $Y$ ) following Poisson distribution, the probability of having observation on  $Y = y$  (say 118 death cases in Table 1) is written as follows:

$$\Pr(Y = y; \mu) = \frac{e^{-\mu} \mu^y}{y!}, \quad y = 0, 1, 2, \dots, \mu > 0. \tag{A-1}$$

The Poisson model can be extended to the zero-inflated Poisson (ZIP) model to account for the zero part (non-progressive BC), including the completely cured and over-diagnosis as diagrammed in Figure 1. By introducing the mixture probability, say  $\pi$ , of being non-progressive (zero-part) extended from (1), the ZIP is specified by:

$$\Pr(Y = y; \mu, \pi) = \begin{cases} \pi + (1 - \pi)e^{-\mu} & \text{when } y = 0 \\ (1 - \pi) \frac{e^{-\mu} \mu^y}{y!} & \text{when } y = 1, 2, 3 \dots \end{cases} \tag{A-2}$$

$$\mu > 0, 0 \leq \pi \leq 1$$

The probability of having the observation on breast cancer death ( $y = 1, 2, \dots$ ) for progressive BCs is thus  $(1 - \pi) \frac{e^{-\mu} \mu^y}{y!}$ , subject to the premise that the women must belong to the progressive breast cancer type with the probability specified by  $(1 - \pi)$ . For women who would not die from breast cancer ( $y = 0$ ), there are two possibilities, the zero part (complete cure and over-diagnosis) and progressive ones but who haven't died at the specified follow-up time. The former is specified by the probability  $\pi$  and the latter is thus the product of the complement scenario and survival probability written as  $(1 - \pi) e^{-\mu}$ . Based on such a specification of the ZIP model, the effect of detection mode denoted by two dummy variables, SD and IC as follows:

$$\text{logit}(\pi) = \log\left(\frac{\pi}{1 - \pi}\right) = \gamma_0 + \gamma_1 SD + \gamma_2 IC \tag{A-3}$$

For a screen-detected subject, the vector of covariate is specified as ( $SD = 1, IC = 0$ ) and the vector of ( $SD = 0, IC = 1$ ) is thus for an interval cancer case. Due to our use of refuser as the reference group, the covariate vector of ( $SD = 0, IC = 0$ ) is specified for such type of case. The probability for being zero (non-progressive BC) is thus:

$$\pi_i = \frac{\exp(\gamma_0 + \gamma_1 SD_i + \gamma_2 IC_i)}{1 + \exp(\gamma_0 + \gamma_1 SD_i + \gamma_2 IC_i)} \tag{A-4}$$

The probability of being zero among all BCs without considering the covariate of detection mode was estimated as 56.14% using only the intercept term. Following the same rationale, the probability of being zero (non-progressive breast cancer) with the incorporation of detection mode was estimated as 66.42% for screen-detected, 50.50% for interval cancer, and 45.40% for cancers form non-participants or outside screening. According to the Equation (1) and (2) in the text of statistical section, 8.94% and 2.86% were estimated for over-diagnosis resulting from mammography and enhanced awareness, respectively.

In order to compare two cumulative survival curves as shown in Figure 2, we need to derive two annual death rates by using the ZIP model ( $\lambda$ ) and the conventional Poisson model ( $\lambda'$ ). For the ZIP model, number of breast cancer death is originated from the non-zero part with the following regression form:

$$\log(\mu) = \log(PY) + \beta_0 + \beta_1x_1 + \beta_2x_2 \dots + \beta_px_p \quad (\text{A-5})$$

The average counts of breast cancer death, say  $\mu$ , which can be further decomposed into the product of death rate ( $\lambda$ ) and the person-year of the breast cancer cases under follow up ( $PY$ ), is written as  $\mu = \lambda \times PY$ . By using log link, the association between average number of breast cancer death and breast cancer death rates and observed person-years is decomposed into  $\log(\mu) = \log(\lambda) + \log(PY)$ . Breast cancer cases with certain characteristics such as large tumor size, higher grade of malignancy, positive lymph node involvement, triple negative cancer, etc., may have unfavorable prognosis and a higher rate of progression to breast cancer death. We denote these characteristics including demographic factors, tumor attributes, IHC markers, type of treatments and detection modes of a breast cancer case by vector  $X$  ( $X = (X_1, X_2, X_3, \dots, X_p)$ ). The effect of these  $P$  characteristics on breast cancer case fatality rate can be incorporated by using log function written as follows:

$$\log(\lambda) = \beta_0 + \beta_1x_1 + \beta_2x_2 \dots + \beta_px_p, \quad (\text{A-6})$$

Based on the adjusted death rate derived from equation (A-6), the prognosis-adjusted survival with the ZIP model is derived by using  $S(t) = \exp\{-\lambda \times t\}$  where  $t$  is the follow-up time (in years) and  $S(t)$  is the survival function for cumulative survival making allowance for overdiagnosis.

A similar logic can be applied to deriving prognosis-adjusted annual death rates ( $\lambda'$ ) using the conventional Poisson regression model without considering the zero-inflated part in the light of  $S'(t) = \exp\{-\lambda' \times t\}$  where  $t$  is the follow-up time (in years) and  $S'(t)$  is the survival function. The comparison of prognosis-adjusted 15-year cumulative survival between the model with and without adjusting for over-diagnosis was made and plotted in the Figure 2 of the main text.

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Article

# Mastectomy or Breast-Conserving Therapy for Early Breast Cancer in Real-Life Clinical Practice: Outcome Comparison of 7565 Cases

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**Abstract:** Although the organ preservation strategy by breast-conserving surgery (BCS) followed by radiation therapy (BCT) has revolutionized the treatment approach of early stage breast cancer (BC), the choice between treatment options in this setting can still vary according to patient preferences. The aim of the present study was to compare the oncological outcome of mastectomy versus breast-conserving therapy in patients treated in a modern clinical setting outside of clinical trials. 7565 women diagnosed with early invasive BC (pT1/2pN0/1) between 1998 and 2014 were included in this study (median follow-up: 95.2 months). In order to reduce selection bias and confounding, a subgroup analysis of a matched 1:1 case-control cohort consisting of 1802 patients was performed (median follow-up 109.4 months). After adjusting for age, tumor characteristics and therapies, multivariable analysis for local recurrence-free survival identified BCT as an independent predictor for improved local control (hazard ratio [HR]:1.517; 95%confidence interval:1.092–2.108,  $p = 0.013$ ) as compared to mastectomy alone in the matched cohort. Ten-year cumulative incidence (CI) of lymph node recurrences was 2.0% following BCT, compared to 5.8% in patients receiving mastectomy ( $p < 0.001$ ). Similarly, 10-year distant-metastasis-free survival (89.4% vs. 85.5%,  $p = 0.013$ ) was impaired in patients undergoing mastectomy alone. This translated into improved survival in patients treated with BCT (10-year overall survival (OS) estimates 85.3% vs. 79.3%,  $p < 0.001$ ), which was also significant on multivariable analysis ( $p = 0.011$ ). In conclusion, the present study showed that patients treated with BCS followed by radiotherapy had an improved outcome compared to radical mastectomy alone. Specifically, local control, distant control, and overall survival were significantly better using the conservative approach. Thus, as a result of the present study, physicians should encourage patients to receive BCS with radiotherapy rather than mastectomy, whenever it is medically feasible and appropriate.

**Keywords:** breast cancer; breast-conserving therapy; mastectomy; outcome; comparative effectiveness

## 1. Introduction

In the early 1980s, large randomized studies first proved that breast-conserving surgery (BCS) followed by postoperative radiotherapy (breast-conserving therapy, BCT) was a valid therapeutic alternative to radical mastectomy in women with early breast cancer (BC) [1,2]. Nowadays, in this setting, breast organ preservation by BCT has become the treatment of choice, due to the excellent outcome and optimal tolerability. Nevertheless, various population-based studies showed that mastectomy is still considered a concrete treatment option and continues to be chosen by several patients with BC in daily clinical practice [3–6].

Treatment of early-stage breast cancer can be considered as a preference-sensitive setting, where decision-making between treatment options can change according to patient preferences [7]. Typical factors able to influence the therapeutic choice in favor of mastectomy include: (i) concerns regarding cancer recurrence, (ii) perception that health outweighs breast retention [8], or (iii) perceived consequences of BCT, including potential adverse effects of radiation therapy [7,9]. Moreover, a renewed interest and trend towards mastectomy has recently emerged, with an increased use of skin-sparing or nipple-sparing mastectomies with immediate breast reconstruction [10–12]. This treatment strategy provides superior aesthetic and quality-of-life outcomes when compared to radical mastectomy. Nevertheless, long-term oncologic outcomes of these new surgical approaches are currently not provided and only retrospective studies were available as evidence. Moreover, the demand for more radical surgical therapies has recently gained wide public attention, as prophylactic mastectomy in BRCA gene mutation positive celebrities attracted notable media interest [13]. The prominence of this issue in the media might also have influenced oncologic patient's preferences regarding their choice of surgical management. In fact, shared decision-making in daily clinical practice is strongly influenced by a number of confounding factors, including clinician preferences and trade-offs regarding toxicity risks or comorbidities [14].

BC management has changed dramatically over time, and local recurrence rates after BCT have decreased significantly [15]. The impact of mammography screening in downward stage migration resulted in smaller tumor sizes and less extensive nodal involvement and is accompanied by improvements in adjuvant treatments, tailored to disease biology.

Therefore, the aim of the present study was to compare the oncological outcome of mastectomy versus breast-conserving therapy in patients treated in “real life”, in a modern clinical setting outside of clinical trials.

## 2. Results

### 2.1. Baseline Patient Characteristics

The final study cohort consisted of 7565 women and the subgroup analysis of the matched cohort included 1802 patients. Patient and treatment characteristics for both cohorts are summarized in Table 1. Overall, 84.8% (6412/7565) of patients were treated with BCS and postoperative RT, while 15.2% (1153/7565) received mastectomy. A significant decrease of mastectomy was documented over time. While in 1998 approximately 21% of patients received a mastectomy, the proportion decreased to 12% in 2014.

Patients treated with BCT were significantly younger, with a median age at diagnosis of 58.2 years in the BCT group, as compared to 59.3 years in the mastectomy group ( $p < 0.001$ ). Furthermore, patients treated with mastectomy presented with more high-risk features such as tumor size  $\geq 20$  mm (43.1%), positive lymph nodes (31.4%), high tumor grade (27.3%) and negative hormone receptor status (12.0%) than patients receiving BCT. Moreover, mastectomy patients received less adjuvant endocrine therapy (52.0% vs. 43.5%,  $p < 0.001$ ). In the matched cohort, we controlled for all these imbalances.

**Table 1.** Patient and tumor characteristics for the entire cohort and the case control cohort.

Variable	Entire Cohort (n = 7565)				p-Value	Case Control Cohort (n = 1802)				
	BCS + RT		Mastectomy			BCS + RT		Mastectomy		
	n	(%)*	n	(%)*		n	(%)*	n	(%)*	
All	6412	(84.8)	1153	(15.2)		929	(50.0)	929	(50.0)	
Age at diagnosis					<0.001					n.s.
<40 years	353	(5.5)	102	(8.8)		68	(7.5)	62	(6.9)	
40–49 years	1193	(18.6)	221	(19.2)		201	(22.3)	184	(20.4)	
50–59 years	1880	(29.3)	282	(24.5)		241	(26.7)	234	(26.0)	
60–69 years	2043	(31.9)	258	(22.4)		215	(23.9)	225	(25.0)	
>70 years	943	(14.7)	290	(25.2)		176	(19.5)	196	(21.8)	
median (years)	58.2		59.3			58.6		58.8		
Lateralisation					0.007					n.s.
right	3181	(49.6)	522	(45.3)		414	(45.9)	414	(45.9)	
left	3231	(50.4)	631	(54.7)		487	(54.1)	487	(54.1)	
Tumour size					<0.001					n.s.
pT1	4790	(74.7)	656	(56.9)		514	(57.0)	514	(57.0)	
pT2	1622	(25.3)	497	(43.1)		387	(43.0)	387	(43.0)	
Nodal status					<0.001					n.s.
pN0	4904	(76.5)	791	(68.6)		646	(71.7)	646	(71.7)	
pN+ (1–3 LN)	1508	(23.5)	362	(31.4)		255	(28.3)	255	(28.3)	
Tumor stage					<0.001					n.s.
T1N0	3860	(60.2)	492	(42.7)		395	(43.8)	395	(43.8)	
T2N0	1044	(16.3)	299	(25.9)		251	(27.9)	251	(27.9)	
T1N1	930	(14.5)	164	(14.2)		119	(13.2)	119	(13.2)	
T2N1	578	(9.0)	198	(17.2)		136	(15.1)	136	(15.1)	
Resection status					n.s.					n.s.
R0	5769	(98.1)	922	(98.2)		812	(98.5)	740	(98.5)	
R1/R2	112	(1.9)	17	(1.8)		12	(1.5)	11	(1.5)	
[unknown]	531	(8.2)	214	(18.5)		77	(8.5)	150	(16.6)	
Grade					<0.001					n.s.
G1	1248	(19.9)	98	(9.1)		77	(8.5)	77	(8.5)	
G2	3610	(57.6)	684	(63.6)		599	(66.5)	599	(66.5)	
G3/4	1411	(22.5)	294	(27.3)		225	(25.0)	225	(25.0)	
[unknown]	143	[2.3]	77	[6.6]						
Hormone receptor					0.038					n.s.
positive	5674	(90.2)	986	(88.0)		83	(9.2)	83	(9.2)	
negative	613	(9.8)	135	(12.0)		818	(90.8)	818	(90.8)	
[unknown]	125	[1.9]	32	[2.7]						
Chemotherapy					n.s.					n.s.
no	4581	(71.4)	855	(74.2)		660	(73.3)	660	(73.3)	
yes	1831	(28.6)	298	(25.8)		241	(26.7)	241	(26.7)	
Endocrine therapy					<0.001					n.s.
no	3076	(48.0)	651	(56.5)		485	(53.8)	485	(53.8)	
yes	3336	(52.0)	502	(43.5)		416	(46.2)	416	(46.2)	

\* Percentages of the presented subcategories are related to the sum of each item with available data; missing values are not taken into account. Hormone receptor positive: estrogen and/or progesterone positive (>1%). BCS: breast conserving surgery, RT: radiotherapy, n.s.: not significant.

## 2.2. Outcome

The median follow-up for the entire cohort was 95.2 months (95%CI: 92.5–97.9) and 109.4 months (95% CI: 104.3–114.5) for the matched cohort. Of the 7565 BC patients, 521 (6.9%) developed local recurrences, 160 (2.1%) lymph node recurrences, and 607 (8.0%) distant metastases.

The cumulative incidence of local recurrence (LR) for the BCT group was 3.2% after 5 years and 8.2% after 10 years. In contrast, the mastectomy group had significantly higher local failure rates with 5.0% 5-year LR and 12.6% 10-year LR rates, respectively ( $p < 0.001$ , Table 2).



**Table 2.** Cumulative incidence of local recurrences (LR) and lymph node recurrences (LNR) and Kaplan-Meier estimates of distant recurrence-free survival (DRFS) and overall survival (OS) for patients of the different cohorts. BCS + RT: breast-conserving surgery with postoperative radiotherapy; Mastectomy: mastectomy without radiotherapy; y: years.

Outcome	Treatment Modality	Entire Cohort			Case Control Cohort		
		Diagnosis 1998–2014 7565 Patients			Diagnosis 1998–2014 1802 Patients		
		5 y (%)	10 y (%)	<i>p</i>	5 y (%)	10 y (%)	<i>p</i>
LR	BCS + RT	3.2	8.2	<0.001	4.6	9.4	0.025
	Mastectomy	5.0	12.6		4.8	12.9	
LNR	BCS + RT	0.9	2.2	<0.001	0.7	2.0	<0.001
	Mastectomy	2.6	5.7		2.5	5.8	
DRFS	BCS + RT	94.5	90.2	<0.001	93.8	89.4	0.013
	Mastectomy	92.0	84.8		93.1	85.5	
OS	BCS + RT	95.2	86.7	<0.001	93.8	85.3	<0.001
	Mastectomy	90.5	77.6		92.2	79.3	

Multivariable Cox analysis for local recurrence-free survival identified mastectomy (hazard ratio [HR], 1.476; 95% confidence interval [CI], 1.164–1.872,  $p < 0.001$ ) as a significant predictor for local failure. Table 3 summarizes other classic prognostic risk factors that had a significant impact on LR risk at multivariable analysis, including young age <40 years ( $p < 0.001$ ), higher tumor stage ( $p < 0.001$ ), high tumor grade ( $p < 0.001$ ) and negative hormone receptor status ( $p = 0.012$ ). In the matched cohort, type of local treatment, age at diagnosis, and tumors stage confirmed their significant impact on LR risk estimates.

**Table 3.** Multivariable Cox regression analysis for local recurrence free survival.

Variable	Entire Cohort ( <i>n</i> = 7565) Local Recurrence Free Survival			Case Control Cohort ( <i>n</i> = 1802) Local Recurrence Free Survival				
	Hazard Ratio HR	95% CI	<i>p</i> -Value	Hazard Ratio HR	95% CI	<i>p</i> -Value		
Local therapy			<0.001			0.013		
BCS + RT	1			1				
Mastectomy	1.476	1.164–1.872	<0.001	1.517	1.092–2.108	<0.001		
Age at diagnosis			<0.001			<0.001		
<40 years	1			1				
40–49 years	0.931	0.671–1.291		0.802	0.475–1.353			
50–59 years	0.521	0.370–0.732		0.309	0.172–0.554			
60–69 years	0.393	0.274–0.565		0.360	0.199–0.651			
≥70 years	0.357	0.228–0.561		0.168	0.075–0.379			
Tumour stage			<0.001			0.020		
T1N0	1			1				
T2N0	1.177	0.899–1.541		0.916	0.584–1.434			
T1N1	1.147	0.855–1.538		1.014	0.601–1.712			
T2N1	2.091	1.565–2.795		1.969	1.204–3.220			
Resection status			0.604			0.330		
R0	1			1				
R1/R2	0.808	0.360–1.812		1.773	0.560–5.618			
Grade				<0.001				0.320
G1	1				1			
G2	2.063	1.438–2.959	1.719		0.821–3.599			
G3/4	2.415	1.619–3.601	1.526		0.676–3.444			

Table 3. Cont.

Variable	Entire Cohort (n = 7565) Local Recurrence Free Survival			Case Control Cohort (n = 1802) Local Recurrence Free Survival		
	Hazard Ratio HR	95% CI	p-Value	Hazard Ratio HR	95% CI	p-Value
Hormone receptor positive	1		0.012	1		0.104
negative	1.466	1.087–1.975		1.575	0.911–2.721	
Chemotherapy			0.402			0.462
yes	1			1		
no	1.110	0.870–1.417		1.172	0.768–1.789	
Endocrine therapy			0.382			0.955
yes	1			1		
no	0.808	0.360–1.812		1.010	0.706–1.447	

Similarly, lymph node recurrences (LNR) were more frequent in patients undergoing mastectomy only. The cumulative incidence of LNR at 5 and 10 years in the BCT group were 0.9% and 2.2%, respectively, compared to 2.6% and 5.7% in patients receiving mastectomy ( $p < 0.001$ ). This observation was still significant on multivariable analysis. Type of local therapy (mastectomy HR 2.442; 95% CI, 1.675–3.560,  $p < 0.001$ ), higher tumor stage ( $p = 0.006$ ) and high tumor grade ( $p < 0.001$ ) did significantly affect the risk of LNR. Focusing on the impact of the type of local treatment in the matched cohort, mastectomy was also correlated with an increased rate of LNR (HR 1.517; 95% CI, 1.092–2.108,  $p = 0.013$ , Table 4).

Table 4. Multivariable Cox regression analysis for lymph node recurrence-free survival (LNRFS).

Variable	Entire Cohort (n = 7565) Lymph Node Recurrence-Free Survival (LNRFS)			Case Control Cohort (n = 1802) Lymph Node Recurrence-Free Survival (LNRFS)		
	Hazard Ratio HR	95% CI	p-Value	Hazard Ratio HR	95% CI	p-Value
Local therapy BCS + RT	1		<0.001	1		0.013
Mastectomy	2.442	1.675–3.560		1.517	1.092–2.108	
Age at diagnosis			0.025			0.030
<40 years	1			1		
40–49 years	1.795	0.857–3.762		1.758	0.576–5.361	
50–59 years	1.143	0.539–2.423		0.715	0.215–2.376	
60–69 years	1.399	0.661–2.960		0.871	0.262–2.890	
≥70 years	0.603	0.238–1.526		0.286	0.058–1.411	
Tumor stage			0.006			0.331
T1N0	1			1		
T2N0	1.754	1.130–2.724		1.175	0.535–2.584	
T1N1	1.274	0.749–2.168		1.433	0.593–3.463	
T2N1	2.300	1.383–3.825		2.186	0.931–5.134	
Resection status			0.366			
R0	1			1		
R1/R2	0.403	0.056–2.888		NA *		
Grade			<0.001			0.082
G1	1			1		
G2	1.451	0.755–2.787		1.121	0.327–3.840	
G3	3.651	1.841–7.242		2.284	0.623–8.371	
Hormone receptor positive	1		0.120	1		0.973
negative	1.523	0.897–2.586		0.982	0.342–2.819	
Chemotherapy			0.221			0.593
yes	1			1		
no	1.303	0.853–1.990		1.223	0.585–2.557	
Endocrine therapy			0.193			0.702
yes	1			1		
no	0.770	0.520–1.141		0.885	0.475–1.652	

\* NA: not applicable, HR not estimable because no event in the R1/2 group.

Ten-year distant metastasis-free survival (DMFS) in the entire cohort was statistically different in the univariate analysis—with 90.2% DMFS in the BCT group, compared to 84.8% in the mastectomy group ( $p < 0.001$ ). This was also seen in a comparable magnitude in the matched cohort ( $p = 0.013$ ). Overall, patients treated with postoperative radiotherapy after BCS showed improved distant control, independent from other covariates in multivariable Cox regression analysis (mastectomy HR 1.257; 95% CI, 1.006–1.570,  $p = 0.044$ ). Other factors correlated with poor DMFS in this cohort were advanced tumor stage ( $p < 0.001$ ), high tumor grade ( $p < 0.001$ ) and negative hormone receptor status ( $p = 0.050$ ). Also in the matched cohort, the positive effect of BCT on DMFS was observed ( $p = 0.008$ , Table 5).

**Table 5.** Multivariable Cox regression analysis for distant metastasis free survival.

Variable	Entire Cohort ( $n = 7565$ ) Distant Metastasis Free Survival (DMFS)			Case Control Cohort ( $n = 1802$ ) Distant Metastasis Free Survival (DMFS)		
	Hazard Ratio HR	95% CI	$p$ -Value	Hazard Ratio HR	95% CI	$p$ -Value
Local therapy			0.044			0.008
BCS + RT	1			1		
Mastectomy	1.257	1.006–1.570		1.537	1.121–2.107	
Age at diagnosis			0.677			0.053
<40 years	1			1		
40–49 years	0.860	0.608–1.216		0.600	0.351–1.027	
50–59 years	0.826	0.592–1.153		0.497	0.292–0.845	
60–69 years	0.785	0.556–1.106		0.437	0.246–0.777	
$\geq 70$ years	0.891	0.601–1.321		0.592	0.314–1.118	
Tumor stage			<0.001			0.001
T1N0	1			1		
T2N0	1.895	1.489–2.411		1.258	0.820–1.932	
T1N1	1.577	1.196–2.080		1.520	0.933–2.477	
T2N1	3.755	2.930–4.812		2.516	1.608–3.936	
Resection status			0.209			0.587
R0	1			1		
R1/R2	1.445	0.813–2.568		1.377	0.435–4.364	
Grade			<0.001			
G1	0.215	0.141–0.327		NA *		
G2	0.514	0.421–0.629		NA		
G3	1			1		
Hormone receptor			0.050			0.706
positive	1			1		
negative	1.327	1.000–2.586		1.110	0.646–1.907	
Chemotherapy			0.656			0.517
yes	1			1		
no	0.951	0.762–1.186		0.874	0.583–1.312	
Endocrine therapy			0.013			0.174
yes	1			1		
no	0.770	0.627–0.946		0.782	0.549–1.114	

\* NA: not applicable, HR not estimable because no event in the R1/2 group.

Among patients treated with BCS plus RT, 10-year OS estimates were 86.7%, and for those treated with mastectomy 77.6% ( $p < 0.001$ ). In multivariable Cox regression analysis, the use of mastectomy was again independently associated with less favorable outcome, with an HR of 1.268 (95% CI, 1.055–1.525,  $p = 0.011$ ). Further risk factors correlated with poor OS in this cohort were older age ( $p < 0.001$ ), advanced tumor stage ( $p < 0.001$ ), and high tumor grade ( $p < 0.001$ ). This effect could be confirmed in multivariable analysis for the matched cohort, where type of local treatment (mastectomy HR 1.452; 95% CI, 1.124–1.875,  $p = 0.004$ , Table 6), older age ( $p < 0.001$ ), advanced tumor stage ( $p < 0.001$ ), and high tumor grade ( $p = 0.033$ ) were independent risk factors.

**Table 6.** Multivariable Cox regression analysis for overall survival.

Variable	Entire Cohort (n = 7565) Overall Survival (OS)			Case Control Cohort (n = 1802) Overall Survival (OS)		
	Hazard Ratio HR	95% CI	p-Value	Hazard Ratio HR	95% CI	p-Value
Local therapy			0.011			0.004
BCS + RT	1			1		
Mastectomy	1.268	1.055–1.525		1.452	1.124–1.875	
Age at diagnosis			<0.001			<0.001
<40 years	1			1		
40–49 years	1.011	0.674–1.517		0.439	0.240–0.804	
50–59 years	1.273	0.870–1.861		0.599	0.346–1.038	
60–69 years	1.757	1.203–2.565		0.854	0.494–1.476	
≥70 years	4.552	3.089–6.710		2.335	1.342–4.065	
Tumor stage			<0.001			<0.001
T1N0	1			1		
T2N0	1.763	1.446–2.150		1.633	1.175–2.270	
T1N1	1.529	1.214–1.925		1.375	0.887–2.130	
T2N1	2.892	2.337–3.580		2.589	1.786–3.753	
Resection status			0.608			0.712
R0	1			1		
R1/R2	1.144	0.685–1.911		1.184	0.484–2.896	
Grade			<0.001			0.033
G1	1			1		
G2	1.406	1.100–1.798		1.968	1.028–3.768	
G3	2.165	1.645–2.848		2.432	1.227–4.820	
Hormone receptor			0.076			0.606
positive	1			1		
negative	1.254	0.986–1.612		1.135	0.702–1.834	
Chemotherapy			0.481			0.708
yes	1			1		
no	1.075	0.880–1.313		1.075	0.736–1.570	
Endocrine therapy			0.662			0.709
yes	1			1		
no	1.039	0.876–1.232		0.946	0.708–1.26	

### 3. Discussion

The present study showed that patients treated with BCS followed by radiotherapy (RT) in a population reflecting “real life” in this clinical setting, had an improved outcome regarding local control, distant control, and overall survival compared to those who underwent a mastectomy. These findings were also confirmed in the matched cohort after adjusting for confounders.

The results presented here are in line with those of other studies investigating the same clinical setting. A population-based analysis of van Maaren et al. [4] of 37,207 breast cancer patients treated in the Netherlands between 01/2000 and 12/2004 obtained similar results. BCS was associated with a significantly improved 10-year overall survival (HR 0.81, 95% CI: 0.78–0.85,  $p < 0.0001$ ). Furthermore, in a representative cohort of patients diagnosed in 2003, BCT had a significant impact on relative survival (HR 0.76, 95% CI: 0.64–0.91,  $p = 0.003$ ). In contrast to the present analysis, distant metastasis-free survival (HR 0.88, 95% CI: 0.77–1.01,  $p = 0.070$ ) was not significantly different in the Dutch cohort, with exception of the T1N0 subgroup (HR 0.60, 95% CI: 0.42–0.85,  $p = 0.004$ ). Yet, in the present analysis, the occurrence of lymph node metastases and distant metastases were both decreased in patients treated with breast-conserving surgery and radiotherapy compared to mastectomy.

Although locoregional and distant control rates are lacking in most of the published experiences [16–19], two studies addressed these issues in a similar BC cohort as in the present study [20,21]. An analysis of the prospective Swedish Multicenter Cohort Study including 2767 patients compared BCS with postoperative RT and mastectomy without RT. Similar to the present analysis, the axillary recurrence-free survival rate at 13 years was significantly reduced after mastectomy without

irradiation as compared to BCS (98.3% versus 96.2%,  $p < 0.001$ ) [21]. Moreover, locoregional recurrence was a strong independent predictor of breast cancer death, (HR: 4.28, 95% CI: 2.55–7.17) and overall survival (HR: 2.64, 95% CI: 1.66–4.19).

The axillary recurrence rates decrease after BCS with RT in comparison to mastectomy, which may have different explanations. Of note, in the present study, 23.5% of patients treated in the BCT group had a positive nodal status with 1–3 involved lymph nodes. While after the AMAROS trial [22], the debate about the role of regional nodal irradiation continues, results from the ACOSOG Z0011 trial [23] and the IBCSG 23-01 trial [24] demonstrated that patients with low-volume nodal disease who are treated with BCS and whole breast RT, can safely avoid axillary lymph node dissection without affecting locoregional control or survival rates [25]. The potential rationale behind this observation is that radiation originating from whole-breast tangential field RT after BCS could exert some protective effect on axillary recurrence rates by controlling the minimal residual disease [21]. It is noteworthy, that the dose to axillary lymph node levels I and II usually is significantly lower than the prescribed dose and can range from 5% to 80% of the prescribed dose (mean value 48.7%). Even in patients receiving regional nodal irradiation of 50 Gray (Gy) to the supra-/infraclavicular lymph node levels (corresponding to levels IV, III and interpectoral lymph nodes), level I receives a reduced dose coverage of mean 41.3 Gy [26,27]. The potential influence of whole breast irradiation, especially in cases of pN+, needs further evaluation in randomized studies.

Regarding distant control, in a single center experience of 6137 cases, Wang et al. [20] observed that patients undergoing BCS plus RT showed a significantly increased 5-year metastasis-free survival ( $p < 0.003$ ) and overall survival ( $p < 0.036$ ) compared with mastectomy. But how could these results be interpreted? Is RT able to add something more than just improved locoregional control? The EBCTCG meta-analysis [28] proved a concrete direct relationship between improved local control and favorable breast cancer specific survival outcome. Nevertheless, the underlying biological mechanisms remain unclear. The oncological community has generated various hypotheses regarding the heterogeneous biology of BC and the impact of available treatment options. A commonly accepted hypothesis is that the addition of RT represents an effective curative treatment for a selected subset of patients who would otherwise have relapsed locally and subsequently would have developed metastases. The fact that the survival benefit only occurs in the framework of successful local control, indicates that RT is involved in events occurring within the treated radiotherapy fields. RT prevents local recurrences through the successful eradication of residual tumor clones or tumor cell clusters within the breast, which are not detected at primary diagnosis. Regarding the beneficial effect on distant tumor control, this interpretation assumes that the metastatic process consists of different waves of cell migration and metastases with differing invasive properties [29]. Hence, RT appears to have unique biological effects to prevent early distant dissemination of cancer cells to distant organs. Furthermore, several potential interactions with the immune system are advocated, including radiation-induced tumor-specific immunity capable of rejecting the colonizing clonogenic cells [29]. Nevertheless, it remains challenging to assess the relative contribution of the interactions between systemic and locoregional treatments on the outcome, as well as that of the individual drugs and RT volumes [15].

The present results of patients treated in a “real life” clinical setting are different from those reported from historical randomized trials of the 1980s, which described similar survival for BCT and mastectomy [30,31]. A key to interpreting these different findings could be that the management of breast cancer has changed considerably over time. Fisher et al. [31] documented a 5-year local recurrence rate of 7.7% for the BCT group and 14.3% in the twenty-year follow-up of the NSABP trial B-06. The 5-year and 10-year cumulative incidence of local recurrences in the present analysis were 3.2% and 8.2%, respectively, suggesting improved local control rates with modern breast cancer therapy, even in the setting outside of randomized trials. The modern multimodal treatment approach, including diagnostics, surgery, systemic therapy, and RT procedures, has improved significantly over the last decades and might explain the survival difference in patients treated with breast-conserving surgery plus radiotherapy as compared to patients treated with mastectomy. [15] The 10-year overall

survival was significantly improved for patients receiving BCS plus radiotherapy: 86.7% with BCT and 77.6% with mastectomy alone ( $p < 0.001$ ). This difference was also observed in the matched cohort ( $p < 0.001$ ). Improved breast cancer specific and overall survival have been found in several population-based cohorts studies [3,5,32–37]. Regarding early-stage breast cancer, Hwang et al. [32] analyzed patients diagnosed with stage I or II breast cancer between 1990 and 2004 and reported improved OS and DSS compared to patients with mastectomy (adjusted hazard ratio for OS entire cohort = 0.81, 95% CI: 0.80–0.83). A registry-based study in Norway also showed comparable results to the present analysis [5]. In multivariate analysis, patients who underwent mastectomy for T1-2/N0-1 BC had an adjusted hazard ratio of 1.65 (95% CI: 1.50–1.82) for OS compared to those who underwent BCT. Similarly, in the present matched cohort, the outcome in terms of OS for patients receiving BCS plus RT was improved (HR: 1.452, 95%CI: 1.124–1.875). Onitilo et al. [35] also compared BCS ± RT versus mastectomy. While overall survival was similar for BCS alone and mastectomy, BCS plus radiation was superior compared to mastectomy alone. The authors concluded, that the survival benefit was not only related to the surgical approach itself but that the addition of adjuvant RT results in a prognostic advantage of BCS plus radiation over mastectomy [35].

We controlled for all variables available in the registry. Unfortunately, we could not account for host-related factors like comorbidities, performance status, or clinician- and patient-related preferences, which may have influenced the clinical treatment decision-making process. It is known that older patients or patients with comorbidities often receive non-standard treatments. A recent analysis of 7581 early stage BC patients diagnosed in 9 European countries analyzed the influence of comorbidities on receiving standard treatments and found that mastectomy was mainly given to elderly women and women with comorbidities [38]. There are several other limitations to effectiveness research due to unpredictable confounding factors and consequently, misinterpretations of treatment and mortality effects should be avoided [39]. In fact, the present observational study may suffer from a “confounding by severity” [40], considering that the severity of the disease (e.g., high-risk factors, tumor biology) could be a potential confounder influencing the indication for mastectomy. Furthermore, survival estimates might be affected by non-tumor-related factors such as age or comorbidities, which could lead to more non-breast cancer deaths. However, we conducted a matched cohort analysis to directly address these concerns and control for these imbalances. Finally, we want to underline that patients of the present study were treated at two specialized breast cancer centers, which in general could have improved outcomes as compared to other settings.

Many previous studies have performed similar analyses with comparable results [4,20,21]. However, a specific characterizing element of the present study is the additional matched case control analysis. This methodology was not used in previously published experiences, which further strengthens the evidence that breast-conserving therapy should be the preferred treatment for patients with early-stage breast cancer when it is medically feasible and appropriate. More specifically, in the present study, we could show that breast-conserving therapy had improved outcome regarding local control, distant control and overall survival as compared to mastectomy alone—even in the matched cohort. Patients were matched regarding a number of variables in order to reduce confounding: 1:1 match for tumor lateralization, tumor size, lymph node status, tumor grade, hormone receptor status, administration of chemotherapy/endocrine therapy and age match with a tolerance of  $\pm 2$  years for age at diagnosis. This resulted in a cohort, where for each mastectomy patient an exactly matched BET patient with exactly the same tumor formula and treatment history was present. Even in this matched cohort analysis, the effect of the choice of surgical treatment on oncologic outcome remained statistically significant.

Since many people still believe that mastectomy may be a better choice, we recommend generating more external validity, such as this retrospective study, in order to gain wide public attention. It is well known that there are a number of barriers to compliance with treatment recommendations, including lack of outcome expectancy. If a physician believes that a treatment will not lead to an improved outcome, he is less likely to follow the treatment recommendations. Another explanation for

the widespread underuse of treatments that were beneficial in controlled trials could be the lack of consideration of external validity [14]. In general, we hope that the present study contributes to the existing evidence regarding the effectiveness of BCS and radiotherapy in this setting.

#### 4. Materials and Methods

For the present analysis, all female patients with a first primary unilateral invasive breast cancer diagnosed between 1998 and 2014 and treated at two Breast Cancer Centers (Red Cross Hospital or LMU Munich, München, Germany) were identified. All data were retrieved from the Munich Cancer Registry. The cancer registry routinely collects data on patient's demographics, primary tumor site, the extent of disease (TNM), histology, treatment, and follow-up. Survival information was obtained systematically through death certificates of health offices. Patients were considered eligible after receiving mastectomy without postoperative radiotherapy (RT) or breast-conserving surgery followed by RT. Patients were excluded if they received neoadjuvant chemotherapy, or in case of histology of ductal carcinoma in situ ( $n = 1.412$ ), lymphoma ( $n = 10$ ) or sarcoma ( $n = 57$ ), or in case of unknown date of initial diagnosis (e.g., tumors from death certificate information only [DCO],  $n = 58$ ). Patients were also excluded if surgery information or pathologic tumor stage was incomplete or missing.

For the present study, women with tumor stages pT1pN0, pT2pN0, pT1pN1 and pT2pN1 (all M0) were selected. For comparison of the standard BCT and mastectomy approaches, we excluded patients with tumor stage  $\geq$ pT3 or more than 3 positive lymph nodes (pN2), as postmastectomy RT (PMRT) would have been routinely recommended in these high-risk patients.

Over the last decades, the use of PMRT was under debate for most intermediate risk patients with small tumor size and limited nodal disease (1–3 positive lymph nodes). Although previous studies provided evidence for a possible survival benefit in intermediate-risk patients [41], PMRT was not uniformly recommended at that time. The standard RT regimen at the Department of Radiation Oncology of the LMU Munich during the observation period was whole-breast irradiation following BCS (50.4 Gy in 28 fractions) using a 3-dimensional conformal tangential field technique with a photon or electron boost of 10–16 Gy to the tumor bed [42].

In order to reduce selection bias and confounding, a subgroup analysis of a matched cohort was performed. To compare treatment outcomes within a set of similar patients, 1:1 case-control matching on the following variables was performed: age at diagnosis, tumor lateralization, tumor size, lymph node status, tumor grade, hormone receptor status, administration of chemotherapy and endocrine therapy. The Hormone receptor was defined positive if estrogen and/or progesterone were positive (>1%).

Statistical analyses were conducted using IBM SPSS Statistics version 24.0 (IBM, Amonk, NY, USA). Frequency data were analyzed using the Chi-Square test. Tolerances values of the case-control matching were set to 2 years for age at diagnosis and 0 (exact matches) for all other above-mentioned variables. Cumulative incidence analysis (CI) was used to calculate the time to LR and LNR and the differences were assessed using the Gray's test for equality of cumulative incidence functions and was performed using R environment for statistical computing and visualization (version. 3.4.0). Distant metastasis-free survival (DMFS) and overall survival (OS) were estimated by the Kaplan-Meier method and tested using the log-rank test. The observation period began after diagnosis of the invasive tumor and ended at the date of distant metastasis occurrence or date of death or the last follow-up for cases without events. In addition, Cox proportional hazards models were used to identify independent prognostic factors related to local recurrence-free survival (LRFs), lymph node recurrence-free survival (LNRFS), DMFS and OS for the different cohorts. The significance level in all analyses was set at 5%.

#### 5. Conclusions

In contrast to the highly selected and homogeneous study populations of randomized trials, this observational analysis included a large patient cohort reflecting “real-life” clinical practice involving a more diverse population, including elderly patients. A fundamental finding of the present study was

that patients treated with BCS followed by RT had improved outcome in clinical practice regarding local control, distant control and overall survival as compared to mastectomy alone. Even if randomized trials provide the least biased estimates to compare treatments and remain the gold standard of efficacy research in oncology, observational data should be appreciated when weighing treatment options for breast cancer surgery. As a result of the present study, it seems advisable to continue to encourage future patients to receive BCS with RT rather than mastectomy when it is medically feasible and appropriate.

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Article

# Trends in Overall Survival and Treatment Patterns in Two Large Population-Based Cohorts of Patients with Breast and Colorectal Cancer

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**Abstract:** Previous studies showed substantial improvement of survival rates in patients with cancer in the last two decades. However, lower survival rates have been reported for older patients compared to younger patients. In this population-based study, we analyzed treatment patterns and the survival of patients with breast cancer (BC) and colorectal cancer (CRC). Patients with stages I–III BC and CRC and diagnosed between 2003 and 2012 were selected from the Netherlands Cancer Registry (NCR). Trends in treatment modalities were evaluated with the Cochran-Armitage trend test. Trends in five-year overall survival were calculated with the Cox hazard regression model. The Ederer II method was used to calculate the five-year relative survival. The relative excess risk of death (RER) was estimated using a multivariate generalized linear model. During the study period, 98% of BC patients aged <75 years underwent surgery, whereas for patients ≥75 years, rates were 79.3% in 2003 and 66.7% in 2012 ( $p < 0.001$ ). Most CRC patients underwent surgery irrespective of age or time period, although patients with rectal cancer aged ≥75 years received less surgery or radiotherapy over the entire study period than younger patients. The administration of adjuvant chemotherapy increased over time for CRC and BC patients, except for BC patients aged ≥75 years. The five-year relative survival improved only in younger BC patients (adjusted RER 0.95–0.96 per year), and was lower for older BC patients (adjusted RER 1.00, 95% Confidence Interval (CI) 0.98–1.02, and RER 1.00; 95% CI 0.98–1.01 per year for 65–74 years and ≥75 years, respectively). For CRC patients, the five-year relative survival improved over time for all ages (adjusted RER on average was 0.95 per year). In conclusion, the observed survival trends in BC and CRC patients suggest advances in cancer treatment, but with striking differences in survival between older and younger patients, particularly for BC patients.

**Keywords:** breast cancer; colorectal cancer; relative survival; older patients; geriatric oncology; cancer treatment

## 1. Introduction

Together with the aging of the population in Western countries, the number of older people with cancer has increased considerably in the last decade [1]. In the Netherlands, breast cancer (BC) is the most common cancer in women, with an estimated incidence rate of 66 new cases per 100,000 women [2]. The incidence of BC is highest in patients aged 70–74 years (212 per 100,000 women) [2]. One of the most common cancers in both men and women is colorectal cancer (CRC), with an estimated incidence of 65 new cases per 100,000 persons. Its incidence increases with age (415 per 100,000 per persons in those aged 75–79 years) [2].

Though the group of older patients has increased considerably, evidence to guide treatment of these patients remains limited [3]. Many clinical trials exclude older patients from participating [4], and older patients who do participate in clinical trials may not be representative of the general older population, as clinical trials often exclude older patients with comorbidities or poor overall health [5].

Several population-based studies showed that the survival rate of BC patients has improved substantially in the last two decades [1,6–8]. This has been attributed particularly to mammographic screening programs [9] and advances in treatment (e.g., improved radiotherapy techniques and new systemic therapeutic agents such as third-generation chemotherapy, aromatase inhibitors, and HER2-targeted therapies). Clinical guidelines, as well as international position papers, state that surgery is recommended for early-stage BC patients [3,10]. Adjuvant chemotherapy is recommended for node-positive or high-risk node-negative disease, and endocrine therapy is recommended for high-risk receptor-positive disease [3,10]. However, population-based studies showed that older BC patients are less likely to receive standard care, which has been linked to lower survival rates in older patients [6,8,11].

Surgery is recommended in early-stage CRC patients. To reduce the risk of recurrence after surgery, clinical guidelines recommend that all lymph node-positive patients should be considered for adjuvant treatment, e.g., radiotherapy, or chemotherapy [12,13]. Several population-based studies showed that the administration of adjuvant chemotherapy has increased considerably in recent years, and that this is associated with improved survival [14,15]. However, the administration of adjuvant chemotherapy is considerably less common in older patients [14].

Several studies state that age should not be a contraindication for surgery or adjuvant chemotherapy [16]. Trends in survival and treatment have been published before, but this has not clarified the differences in survival rates between cancers. The aim of this study was to investigate trends in survival rates and treatment patterns over time for older versus younger patients with BC and CRC in the Netherlands.

## 2. Results

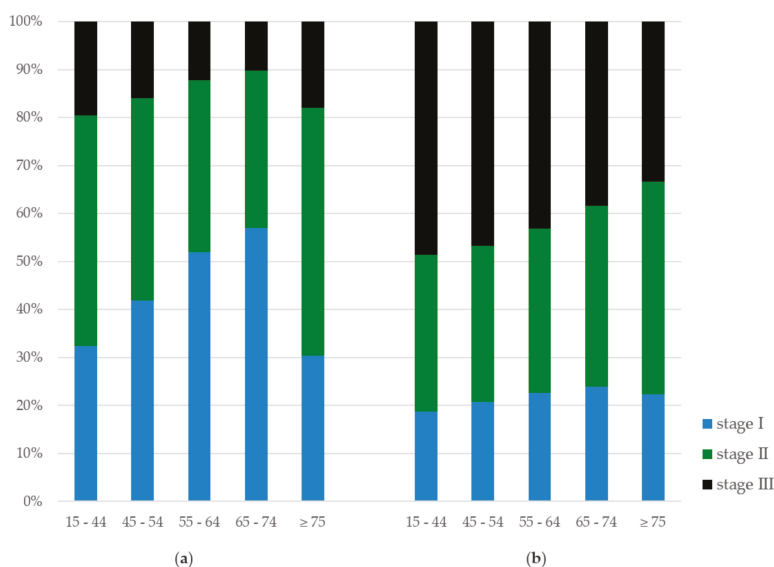
Between 2003 and 2012, 127,146 patients were diagnosed with stages I–III BC in the Netherlands, while 85,629 patients were diagnosed with stages I–III CRC (Table 1). The median age of patients with BC was 60 years (range 18 to 103), and the median age of patients with CRC was 71 years (range 18 to 102).

**Table 1.** Clinical characteristics of 127,146 breast cancer (BC) and 85,629 colorectal cancer (CRC) patients, diagnosed in 2003–2012.

Characteristics	BC		CRC	
	<i>n</i>	%	<i>n</i>	%
Gender				
Male	-		45,783	53.5
Female	127,146	100	39,846	46.5
Age (years)				
15–44	15,206	12	2077	2.4
45–54	30,539	24	6457	7.5
55–64	31,830	25	17,969	21
65–74	27,095	21.3	26,839	31.3
≥75	22,476	17.7	32,287	37.7
Year of diagnosis				
2003	11,669	9.2	7367	8.6
2004	11,848	9.3	7813	9.1
2005	11,845	9.3	7877	9.2
2006	12,264	9.6	8190	9.6
2007	12,725	10	8510	9.9
2008	12,836	10.1	8713	10.2
2009	12,999	10.2	8877	10.4
2010	13,102	10.3	9135	10.7
2011	13,838	10.9	9486	11.1
2012	14,020	11	9661	11.3
Stage				
I	56,579	44.5	19,453	22.7
II	52,049	40.9	33,363	39
III	18,518	14.6	32,813	38.3

### 2.1. Stage at Diagnosis

The stages at diagnosis according to age are presented in Figure 1. BC patients aged  $\leq 45$  or  $\geq 75$  years were more often diagnosed with stage II or III cancer compared to those aged 45–75 years ( $p < 0.001$ ). Stage II was diagnosed in 51.7% and stage III in 17.9% of the BC patients aged  $\geq 75$  years. The percentage of CRC patients with stage II disease steadily increased with age (32.5% in CRC patients aged 45–54 years, and 44.3% in CRC patients aged  $\geq 75$  years), while stage III decreased with age (46.7% in CRC patients aged 45–54 years, and 33.3% in CRC patients aged  $\geq 75$  years,  $p < 0.001$ ).



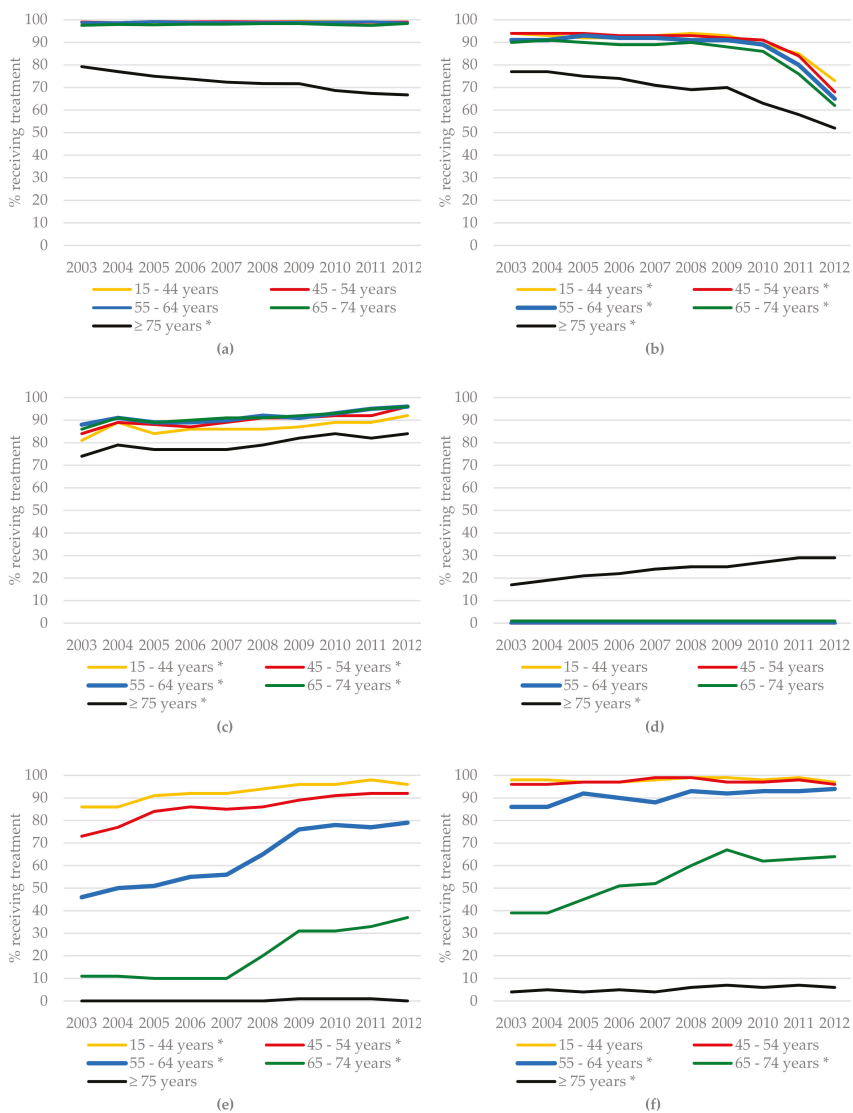
**Figure 1.** Tumor stage by age at diagnosis: (a) breast cancer, and (b) colorectal cancer.

## 2.2. Trends in Treatment

The time trends for all treatments for BC patients between 2003 and 2012 are presented in Figure 2. Over the entire study period, 98.0% of patients with stages I–III BC who were younger than 75 years underwent surgery, whereas for those aged 75 years or over, surgery rates declined from 79.3% in 2003 to 66.7% in 2012 ( $p < 0.001$ , Figure 2a). The proportion of BC patients undergoing lymph node dissection for node-positive disease decreased over time for all age groups (Figure 2b). The use of radiotherapy after breast-conserving surgery increased significantly for all age groups but was lowest among patients aged 75 years and older (73.6% in 2003 vs. 84.1% in 2012,  $p < 0.001$ ) and highest among patients aged 45–54 years (84.4% in 2003 vs. 96.2% in 2012,  $p < 0.001$ , Figure 2c). Primary endocrine therapy as a monotherapy in BC patients was almost exclusively used for patients aged 75 years or over, and increased significantly over time (16.5% in 2003 and 28.7% in 2012,  $p < 0.001$ , Figure 2d). The use of adjuvant chemotherapy in stages II and I BC patients increased significantly over time for all age groups, except for patients aged 75 years or older. Its use was associated with age, with more than 90% of patients younger than 55 years and less than 9% of patients aged 65 years or older in 2012 receiving it, and hardly any patients aged 75 years or older (Figure 2e,f).

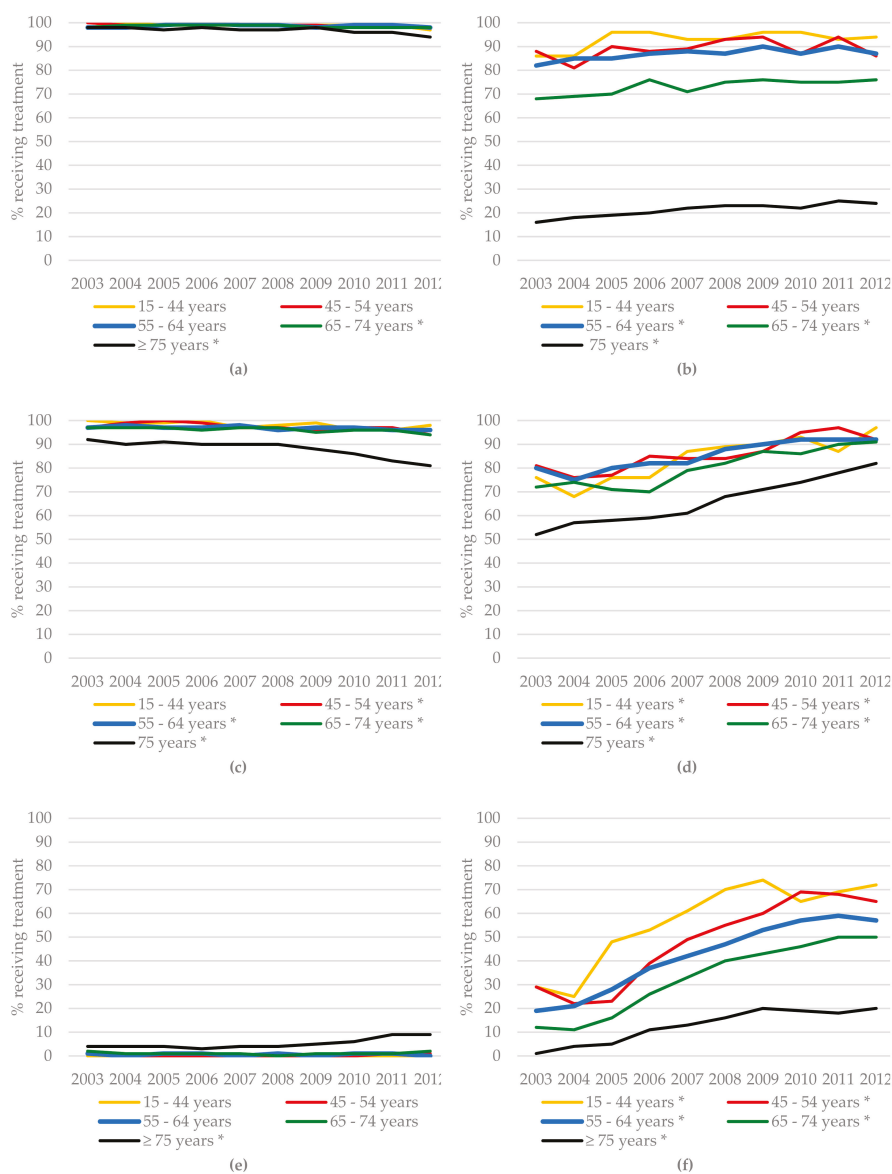
As CRC patients are treated according to tumor location, i.e., colon cancer (CC) or rectal cancer (RC), we report the treatments received for CC and RC separately (Figure 3). In CC, the number of patients aged 75 years or older who underwent surgery declined significantly over time (98.1% in 2003 vs. 94.2% in 2012,  $p < 0.001$ , Figure 3a). The percentage of chemotherapy treatment in stage III CC patients aged 55 years or older increased significantly over time (Figure 3b), although the percentage of those aged 75 years or older who received chemotherapy remained considerably lower (16.3% in 2003 vs. 23.5% in 2012,  $p < 0.001$ ). The percentage of RC patients aged 45 years or older who underwent surgery declined significantly over time, the greatest decrease being observed in RC patients aged 75 years or older (from 91.8% in 2003 to 81.1% in 2012,  $p < 0.001$ ) (Figure 3c). The use of radiotherapy in stage III RC increased significantly over time for all age groups but became less frequent with increasing age (91.8% of patients aged 55–64 years compared to 80.2% of patients aged 75 years or older in 2012, Figure 3d). The number of RC patients aged 75 years or older undergoing surgery was lower than that of younger patients. The percentage of I–III RC patients aged 75 years or older who received

primary radiotherapy as a single treatment modality increased significantly, from 3.8% in 2003 to 9.4% in 2013 ( $p < 0.001$ , Figure 3e). The use of chemoradiation in stage II RC patients increased significantly in all age groups, ranging from 19.8% to 71.6% in 2012, but again this was highly dependent on age, with the lowest use in the age group of 75 years and older (Figure 3f).



**Figure 2.** Time trends in the treatment of breast cancer (BC) patients according to age group: (a) surgery in BC patients stages I–III, (b) lymph node dissection in BC patients stages I–III with node-positive disease, (c) radiotherapy after breast-conserving surgery in BC patients stages I–III, (d) primary endocrine therapy in BC patients stages I–III, (e) chemotherapy in BC patients stage II, and (f) chemotherapy in BC patients stage III. \* Significant ( $p < 0.05$ ) difference in treatment over time using the Cochran–Armitage trend test.





**Figure 3.** Time trends of treatment in colon cancer (CC) and rectal cancer (RC) patients according to age group: (a) surgery in CC patients stages I-III, (b) chemotherapy in CC patients stage III, (c) surgery in RC patients stages I-III, (d) radiotherapy in RC patients stage III, (e) primary radiotherapy as a single treatment modality in RC patients stage III, and (f) chemoradiation in RC patients stages II-III. \* Significant ( $p < 0.05$ ) difference in treatment over time using the Cochran–Armitage trend test.

### 2.3. Trends in Survival

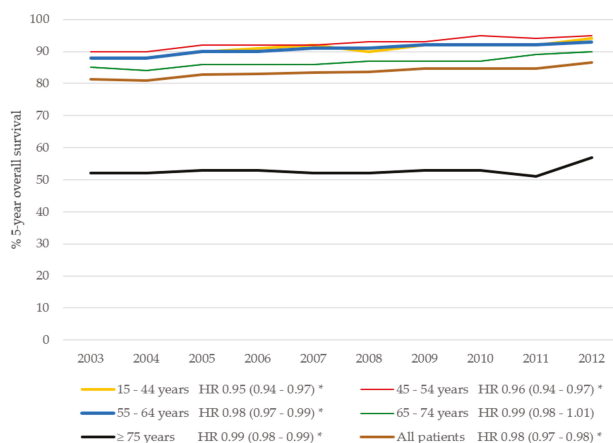
The 5-year overall survival of BC patients is presented in Figure 4. The 5-year overall survival improved in BC patients (adjusted hazard ratio (HR) 0.78, 95% CI 0.73–0.83) in the period 2003–2012. The 5-year overall survival improved slightly in patients aged 75 years or older (adjusted HR 0.99, 95%

CI 0.98–0.99) and did not significantly improve in patients aged 65–74 years (adjusted HR 0.99, 95% CI 0.98–1.01). In the multivariable Cox regression analysis, the overall survival improved in the period 2003–2012 and was influenced significantly by age (Table 2). The 5-year relative survival improved in BC patients, from 89.7% in 2003 to 93.1% in 2012 (adjusted relative excess risk (RER) 0.97, 95% CI 0.97–0.98 per year) (Figure 5). The 5-year relative survival rate improved significantly for BC patients aged 65 years or younger over the period between 2003 and 2012. In contrast, the relative survival for BC patients aged 75 years or older did not increase significantly over the same period (crude RER 0.99, 95% CI 0.97–1.02). After adjustment for age, stage, and treatment, the RER for BC patients aged 65–74 years was no longer significant (adjusted RER 1.00, 95% CI 0.98–1.01), and the RER for patients aged 75 years or older remained non-significant (adjusted RER 1.00, 95% CI 0.98–1.01).

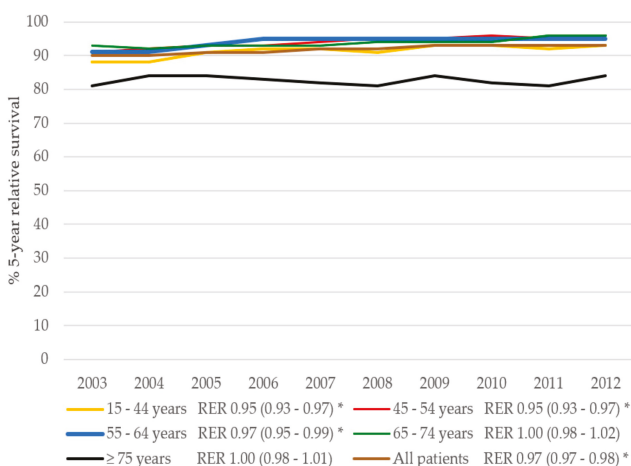
**Table 2.** Multivariate Cox hazard analysis of five-year overall survival.

Variable	BC		CRC	
	HR (95% CI) <sup>a</sup>	<i>p</i>	HR (95% CI) <sup>a</sup>	<i>p</i>
Year of diagnosis (per year)	0.98 (0.97–0.98)	<0.001	0.96 (0.96–0.97)	<0.001
Age		<0.001		<0.001
15–44 years	1.04 (0.97–1.11)		<b>0.87 (0.76–0.98)</b>	
45–54 years	1.00 (ref)		1.00 (ref)	
55–64 years	<b>1.35 (1.28–1.43)</b>		<b>1.25 (1.17–1.34)</b>	
65–74 years	<b>2.06 (1.95–2.18)</b>		<b>1.81 (1.70–1.93)</b>	
≥75 years	<b>4.89 (4.60–5.18)</b>		<b>3.43 (3.22–3.65)</b>	
Gender	-			<0.001
Male	-		1.00 (ref)	
Female			<b>0.82 (0.80–0.84)</b>	
Stage		<0.001		<0.001
I	1.00 (ref)		1.00 (ref)	
II	<b>1.79 (1.72–1.87)</b>		<b>1.50 (1.44–1.55)</b>	
III	<b>4.14 (3.96–4.32)</b>		<b>2.74 (2.64–2.84)</b>	
Grade		<0.001		<0.001
Well differentiated	1.00 (ref)		1.00 (ref)	
Moderately differentiated	<b>1.29 (1.22–1.35)</b>		1.02 (0.97–1.07)	
Poorly differentiated and undifferentiated	<b>2.14 (2.03–2.25)</b>		<b>1.46 (1.38–1.55)</b>	
Unknown	<b>1.67 (1.57–1.77)</b>		<b>1.12 (1.06–1.19)</b>	
Surgery		<0.001		<0.001
No	1.00 (ref)		1.00 (ref)	
Yes	<b>0.30 (0.28–0.32)</b>		<b>0.14 (0.14–0.15)</b>	
Radiotherapy		<0.001		<0.001
No	1.00 (ref)		1.00 (ref)	
Yes	<b>0.63 (0.61–0.65)</b>		<b>0.76 (0.74–0.78)</b>	
Chemotherapy		<0.05		<0.001
No	1.00 (ref)		1.00 (ref)	
Yes	<b>0.95 (0.90–0.99)</b>		<b>0.64 (0.62–0.66)</b>	
Hormone therapy		<0.001		
No	1.00 (ref)		-	
Yes	<b>0.55 (0.53–0.57)</b>			

<sup>a</sup> Adjusted for all variables included in the model. HR, hazard ratio. Significant (*p* < 0.05) HR values are indicated in bold. ref, reference 95% CI, 95% Confidence Interval.

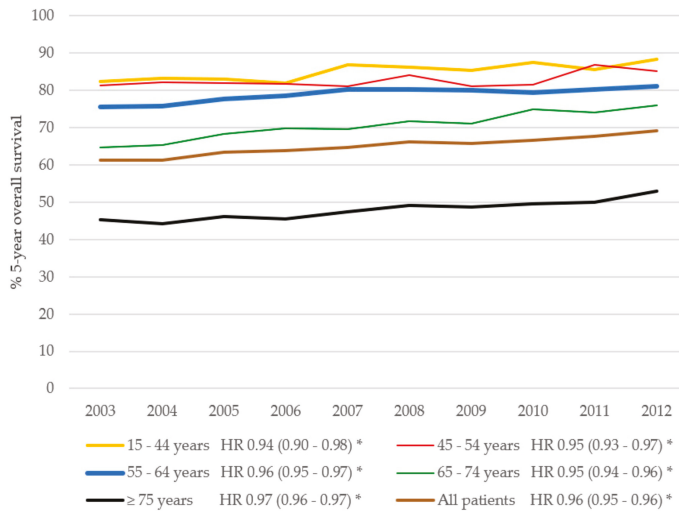


**Figure 4.** Five-year overall survival in BC patients over time per age group. HR indicates hazard ratio per year, adjusted for grade, stage, and treatment. \* Significant ( $p < 0.05$ ).

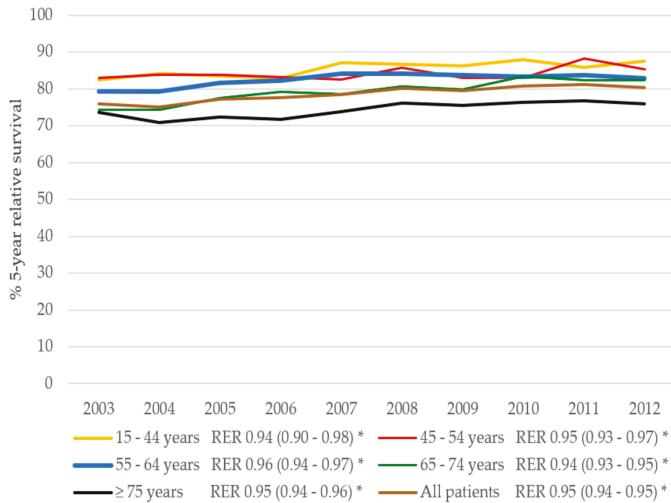


**Figure 5.** Five-year relative survival in BC patients over time per age group. RER indicates relative excess risk per year, adjusted for grade, stage, and treatment. \* Significant ( $p < 0.05$ ).

Similarly, the 5-year overall survival improved for all CRC patients (Figure 6). Overall survival in CRC patients was influenced by the year of diagnoses and age. Other factors affecting 5-year overall survival were, stage, grade, gender, and treatment. The relative survival for CRC patients improved from 76.0% in 2003 to 80.3% in 2012 (adjusted RER 0.95, 95% CI 0.94–0.95 per year, Figure 7). Most overall improvement was observed in the CRC patients aged 65–74 years. In this group, the 5-year relative survival increased by 7.9% between 2003 and 2012 (adjusted RER 0.94, 95% CI 0.93–0.95). In contrast to the BC cohort, the RERs for patients with CRC in all age groups had improved significantly after adjustment for grade, sex, stage, and treatment (adjusted RERs varying between 0.94 and 0.96 per year).



**Figure 6.** Five-year overall survival in CRC patients over time per age group. HR indicates the hazard ratio per year, adjusted for grade, stage, gender, and treatment. \* Significant ( $p < 0.05$ ).



**Figure 7.** Five-year relative survival in CRC patients over time per age group. RER indicates the relative excess risk per year, adjusted for grade, stage, gender, and treatment. \* Significant ( $p < 0.05$ ).

### 3. Discussion

The findings of our population-based study show that 5-year overall and relative survival improved for BC patients and for CRC patients in the Netherlands between 2003 and 2012. The difference in 5-year survival between older and younger CRC patients decreased during the study period, and the greatest improvement in overall and relative survival was observed for older CRC patients. Among BC patients, substantial improvement of 5-year relative survival was seen only for younger BC patients. The overall and relative survival and its improvement over time were lower for older BC patients.

Previous population-based studies found that overall and relative survival among older BC patients did not change in the last decade [17,18]. This lack of improvement has been attributed to differences in the delivery of cancer care between older and younger BC patients, e.g., the influence of comorbid conditions on the selection of cancer treatments [19,20]; the lower likelihood of receiving breast surgery, radiotherapy, or adjuvant chemotherapy for older BC patients compared to younger BC patients [6]; and mammographic screening being offered only to younger women [9]. The survival and treatment differences observed in our study suggest possible limitations of cancer treatment, as well as reticence about cancer treatment, for older BC patients.

The most notable finding was the decreasing proportion of older BC patients receiving surgery, and the increasing proportion receiving primary endocrine therapy. Previous population-based studies in the Netherlands confirm this finding [6,17,21]. Van de Glas et al. showed that the proportion of older BC patients that did not receive breast surgery increased substantially from 9.2% in 1995 to 30.1% in 2011 [17]. Moreover, previous population-based studies showed that the proportion of older BC patients receiving primary endocrine therapy is higher in the Netherlands than in other European countries [22–24]. The EUROCARE breast cancer group found lower survival rates in European countries, like England and Ireland, where older BC patients are treated more often with primary endocrine therapy omitted [22]. This increased use of primary endocrine therapy may have been influenced by a meta-analysis, in which seven randomized controlled trials were included [25]. This meta-analysis showed that, compared to those treated with primary endocrine therapy, surgery alone seemed to have no beneficial impact on the overall survival rate (HR 0.98, 95% CI 0.74–1.30). However, BC patients who underwent surgery with adjuvant endocrine therapy had a borderline significantly improved overall survival rate (HR 0.86, 95% CI 0.73–1.00) and a significantly better disease-free survival. The HR for endocrine therapy alone was 0.65 (95% CI 0.53–0.81) [25].

Recent reports have advocated that primary endocrine therapy should only be considered in frail older BC patients [26]. Guidelines in the Netherlands state that patients with operable tumors should be treated with surgery and not primary endocrine therapy, irrespective of age [10]; also, the International Society of Geriatric Oncology (SIOG) recommends that primary endocrine therapy should only be offered to BC patients with a life expectancy of less than three years, or who are unfit for or refuse surgery [3]. However, as a large proportion of older BC patients are currently being treated with primary endocrine therapy, it is not clear if this recommendation is being followed consistently. In a small retrospective study in the Netherlands, Hamakers et al. found that the reason for not carrying out surgery was not always clearly stated in medical charts. When documented, reasons were often higher age, poor overall health, patient preference, and comorbidities [21]. Additionally, Sierink et al. found in a small cohort in the Netherlands that BC patients are less likely to receive surgery when they are older, have been diagnosed with more comorbidities, or have been diagnosed with a more advanced disease [27]. However, other studies found that age was the most important reason for foregoing surgery in older BC patients, even when adjustments were made for comorbidities [28–30].

Several studies found that the main factor for surgical morbidity in BC patients are comorbidities and not age [31]. Breast surgery can be considered a low morbidity surgery [32]. Approximately one third of BC patients develop breast and/or axillar wound infections, seromas, and hematomas after surgery, depending on the type of surgery. These surgical morbidities are often minor and treated in an outpatient setting [32]. Moreover, van de Glas et al. demonstrated that relative mortality was not influenced by postoperative complications in older BC patients [33].

Our study showed that the proportion of surgery in older CRC patients remains high. Many studies have shown that age should not prohibit surgery in CRC patients [16]. In RC, however, the proportion of patients aged  $\geq 75$  years that received primary chemoradiation increased from 0% in 2003 to almost 20% in 2012. Recently, foregoing surgery after chemoradiation has been shown to be safe for RC patients in whom a complete clinical response is seen [34].

In the present study, the proportion of older BC patients receiving adjuvant chemotherapy was lower than that of younger BC patients. In Europe, different rates of adjuvant chemotherapy in BC

patients have been reported, with lower rates in the Netherlands [22]. There has been conflicting evidence regarding the benefits of this treatment for older BC patients. Data from the Early Breast Cancer Trial demonstrated a decreasing benefit from adjuvant chemotherapy with age [35,36], although relatively few BC patients aged 70 years or older have been included in clinical trials [5]. Nevertheless, the results of this meta-analysis may have contributed to the lower use of adjuvant chemotherapy in older BC patients [35]. Other recent studies suggest a possible benefit from adjuvant chemotherapy in older BC patients, especially in those with HER2-positive disease [37,38]. Recently, Du et al. compared the effectiveness of adjuvant chemotherapy between older BC and CRC patients, and found that chemotherapy might be effective in BC patients up to the age of 79 years [39].

Possible explanations why older BC patients receive adjuvant chemotherapy less often are frailty, poor functional status, or comorbidities. In addition, older patients are less willing to accept possible side effects associated with adjuvant chemotherapy compared with younger patients, and are concerned about negative influences of adjuvant chemotherapy. Most older BC patients receive adjuvant endocrine therapy because they are more likely to have a receptor positive disease, which tends to grow more slowly, are usually well differentiated, and respond better to endocrine therapy. However, there are side effects associated with endocrine therapy, like deep vein thrombosis or pulmonary emboli [40].

The 5-year overall and relative survival improved in older CRC patients in our study. In a population-based study, Van den Broek et al. showed that 5-year relative survival had increased significantly in CC patients aged <65 years and  $\geq 75$  years over the period from 1991 to 2005, but not in patients aged 65–74 years [41]. In our study the greatest overall improvement of relative survival was seen in CRC patients aged 65–74 years (74.4% in 2003 vs. 82.3% in 2012). In the  $\geq 75$  years age group, relative survival improved significantly, but still lagged behind the survival of younger age groups. However, the difference in survival between younger and older CRC patients reported by the EURO-CARE working group [1,7] diminished over time in our study. This improved survival could be partly attributed to the increased use of adjuvant chemotherapy in older patients [14], but also to other factors, such as improved preoperative staging and improved surgery [42].

This study had some limitations. Information on patient characteristics, such as comorbidities, was not available. Furthermore, we can draw no conclusions about aspects such as quality of life, maintaining functional status, risk of recurrence, or complications, as we had no data for these outcomes. Although we adjusted the analyses for potential confounders, there may still have been some confounding by other factors. Finally, we did not have data on the causes of mortality. On the other hand, the use of data for two different tumor types means that our study does provide important insights into the differential treatment and survival trends by age. Another strength of our study is the large number of patients with BC and CRC included from the Netherlands Cancer Registry, with at least 5 years of follow-up.

## 4. Materials and Methods

### 4.1. Patients

For this population-based study, we selected all patients with primary BC and CRC stages I–II diagnosed between 2003 and 2012 from the Netherlands Cancer Registry (NCR). We only selected patients with stages I–III, as this group could potentially be treated with curative intent. Furthermore, only female BC patients were selected. The NCR records all newly diagnosed malignancies in the Netherlands after notification from the nationwide automated pathological archive (PALGA). This data is supplemented with data from the National Registry of Hospital Discharge Diagnosis. Trained registrars from the NCR routinely collect data from medical records regarding patient, tumor, and treatment characteristics in all Dutch hospitals.

Cancer stage was based on the pathological TNM classification applicable at the time of cancer diagnosis. In case of a missing pathological TNM classification, the clinical TNM classification was used instead. Data regarding the vital status of all patients was available through linkage with the

municipal personal records database, with complete follow-up until 31 December 2017. Patients were divided into five groups according to age: 15–44, 45–54, 55–64, 65–74, and 75 years or older. Primary cancer treatment retrieved from the NCR included surgery, radiotherapy, endocrine therapy, immunotherapy, and (neo-)adjuvant chemotherapy.

According to the Central Committee on Research involving Human Subjects (CCMO), this type of study does not require approval from an ethics committee in the Netherlands. The Privacy Review Board of the Netherlands Cancer Registry approved this study in January 2018 (K17.351).

#### 4.2. Statistical Analyses

All analyses were performed in IBM SPSS statistics version 22.0 (SPSS, Chicago, IL, USA) or SAS statistics version 9.4 (SAS institute, Inc., Cary, NC, USA). Statistical significance was defined as  $p \leq 0.05$ .

The Cochran-Armitage trend test was used to test for differences in treatment over time. Treatments are presented as percentages per age group and period.

The Cox hazard analysis was used to calculate the overall survival. The hazard ratio (HR) was calculated according to age group and cancer stage, and adjusted for cancer stage, grade, gender, and treatment.

As cause of death is not available in the NCR, relative survival was used as an estimation of the disease-specific survival, and was calculated using the Ederer II method, i.e., as the observed overall survival of patients in the study divided by the expected overall survival in a matched group of the general population by age, sex, and year [11]. The expected survival was obtained from national life tables. The relative excess risk of death (RER) was estimated using a multivariate generalized linear model with a Poisson distribution. The RER was calculated according to age and cancer type, and adjusted for cancer stage, grade, gender, and treatment. The treatment variables were added to investigate the effect of treatment on the RER.

## 5. Conclusions

In this population-based study analyzing oncological treatment patterns and relative survival of BC and CRC patients, we observed substantial differences between younger and older patients. Although the observed survival trends in BC and CRC patients suggest advances in cancer treatment, we found that the survival of older BC patients in particular was strikingly lower. Moreover, the differences in relative survival between younger and older BC patients revealed in previous studies were found to have continued to increase in recent years. Inequalities in the provision of cancer care to older BC patients need to be investigated in future cancer research. Selection criteria for specific treatments could eventually lead to individualized and optimized treatment for older cancer patients.

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Article

# Liver Kinase B1—A Potential Therapeutic Target in Hormone-Sensitive Breast Cancer in Older Women

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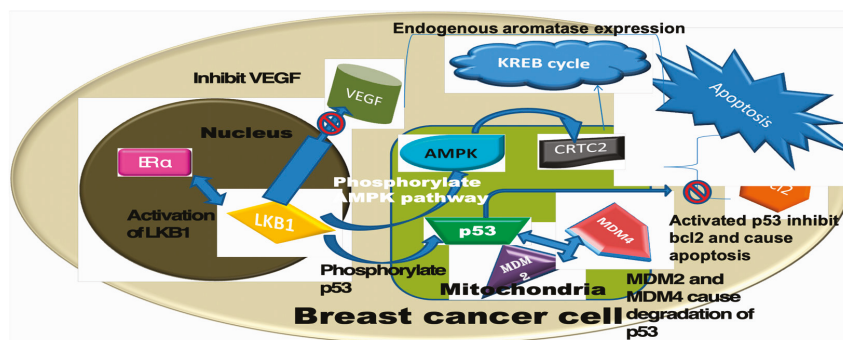
**Abstract:** *Background:* The role of liver kinase B1 (LKB1), a serine/threonine kinase, has been described in the development of PeutzJagher’s syndrome, where a proportion (~45%) of patients have developed breast cancer in their lifetime. Cell line studies have linked LKB1 with oestrogen receptors (ER) and with the Adenosine monophosphate-activated protein kinase (AMPK) pathway for energy metabolism. However, limited studies have investigated protein expression of LKB1 in tumour tissues and its intracellular relationships. This study aimed to investigate the intracellular molecular relationships of LKB1 in older women with early operable primary breast cancer and its correlation with long-term clinical outcome. *Methods:* Between 1973 and 2010, a consecutive series of 1758 older ( $\geq 70$  years) women with T0-2N0-1M0 breast carcinoma were managed in a dedicated facility. Of these, 813 patients underwent primary surgery, and 575 had good quality tumour samples available for tissue microarray construction. LKB1 was assessed in 407 cases by indirect immunohistochemistry (IHC). Tumours with 30% or more of cells with cytoplasmic LKB1 expression were considered positive. LKB1 expression was compared with tumour size, histological grade, axillary lymph node stage, ER, PgR, EGFR, HER2, HER3, HER4, BRCA1&2, p53, Ki67, Bcl2, Muc1, E-Cadherin, CD44, basal (CK5, CK5/6, CK14 and CK17) and luminal (CK7/8, CK18 and CK19) cytokeratins, MDM2 and MDM4, and correlated with long-term clinical outcome. *Results:* Positive LKB1 expression was seen in 318 (78.1%) patients, and was significantly associated with high tumour grade, high Ki67, over-expression of HER2, VEGF, HER4, BRCA2, MDM2 and negative expression of CD44 ( $p < 0.05$ ). There was no significant correlation with tumour size, axillary lymph node status, ER, PgR, p53, basal or luminal cytokeratins, Bcl2, Muc1, EGFR, HER3, MDM4, E-cadherin and BRCA1. LKB1 did not show any significant influence on survival in the overall population; however, in those patients receiving adjuvant endocrine therapy for ER positive tumours, those with positive LKB1 had significantly better 5-year breast cancer specific survival when compared to those without such expression (93% versus 74%,  $p = 0.03$ ). *Conclusion:* LKB1 expression has shown association with poor prognostic factors in older women with breast cancer. However, LKB1 expression appears to be associated with better survival outcome among those patients receiving adjuvant endocrine therapy. Further research is required to explore its potential role as a therapeutic target.

**Keywords:** LKB1; Breast Cancer; Older women; Metformin; Endocrine therapy

## 1. Introduction

Liver kinase B1 (LKB1) or serine/threonine kinase-11, a 436-amino acid chain protein with a molecular weight of 49 kDa, is associated with the development of Peutz-Jeghers syndrome (PJS) [1,2].

In addition, approximately 45% of PJS patients have been reported to develop breast cancer in their lifetime [3]. LKB1 is known to work as a tumour suppressor gene as well as an upstream kinase which is linked to the cellular functions during normal state and in metabolic stress response [4,5]. LKB1 is normally located in the nucleus, however, upon activation, it is relocated to the cytoplasm, where it acts as a co-activator of the oestrogen receptor (ER), controls the citric acid cycle (KREB's cycle) and cell energy metabolism via the adenosine mono-phosphate activated protein kinase (AMPK) pathway, and inhibits the production of the aromatase enzyme [6–11]. It has also been reported to control the cell cycle at the G0–G1 check point via the p53 pathway, to be associated with angiogenesis through the control of Vascular Endothelial Growth Factor (VEGF) and with cell polarity [8,11,12]. A proposed mechanism of LKB1 function in breast cancer cells is presented in Figure 1. Previously, reported data linked LKB1 with poor prognostic factors [11]. A cell line study on MDA-MB-231 cells showed an association of LKB1 with gemcitabine resistance [13]. Additionally, cell line studies suggested that LKB1 is involved in DNA repair, thus deficient cells demonstrate delayed repair and respond well to DNA-based therapy, such as PARP inhibitors [14]. Cell-based studies showed that LKB1-null cells possess invasion and breast cancer stem cell like properties. However, LKB1 enhancing therapy (i.e., Honokoil, a bioactive molecule) showed improving outcome by reducing cellular invasiveness and stemness [15], thus suggesting a therapeutic significance.



**Figure 1.** Proposed intracellular mechanism of action of LKB1 as suggested in the cell line studies [7–9,12,16]. VEGF: Vascular Endothelial Growth factor, KREB: Citric acid cycle, ER: Oestrogen receptor, CRTC2: Creb-regulated transcription co-activator 2, MDM2/4: Mouse double minute 2/4.

Currently available literature is based on in vitro or in vivo model studies, or gene technologies, such as Western blot. However, there is limited information available on the subject of using immunohistochemistry (IHC), which is currently a widely used technique for analysis of therapeutic targets in clinical practice. This study was designed to analyse the association of LKB1 with other biological markers of known significance in breast cancer and to correlate its expression with long-term clinical outcome in older women with primary breast cancer.

## 2. Materials and Methods

### 2.1. Patients

Over 37 years (1973–2010), 1758 older ( $\geq 70$  years) women with early operable primary breast cancer (T0–2, N0–1, M0) were managed in a dedicated facility in a single institution with clinical information available from diagnosis till death/last follow-up. Eight hundred and thirteen patients underwent primary surgery (with optimal adjuvant therapy as per unit policy at the time [17]) and among them, good quality formalin-fixed paraffin-embedded surgical specimens were available from 575 patients for tissue microarray (TMA) construction. The management pattern over this period of time was evolving, a detailed description of which was reported earlier [17]. Briefly during the

1970s and 1980s, ER status was not available and there was no consensus on recommendations for adjuvant systemic therapy. During the 1990s, adjuvant endocrine therapy was advised based on the clinical judgement of the treating physician and/or the patient's choice. In the recent decade, there have been structured recommendations for the use of adjuvant endocrine therapy. Those patients with ER positive tumours and Nottingham Prognostic Index (NPI) <3 (excellent prognostic group) were not given any adjuvant systemic therapy, those with an NPI of 3–3.4 (good prognostic group) were offered endocrine therapy and given the choice of tamoxifen or a non-steroidal aromatase inhibitor, and those with an NPI of >3.4 (moderate to poor prognostic group) were given a non-steroidal aromatase inhibitor. Adjuvant chemotherapy was considered in fit, relatively younger patients with moderate to poor prognostic tumours, in particular those with ER negative tumours. Trastuzumab was considered in relatively fit patients with HER2 positive tumours having moderate to poor prognosis.

## 2.2. Tissue Microarray Construction

Tissue microarrays (TMAs) of formalin-fixed paraffin-embedded tumour sections were constructed as described [18]. Briefly, 0.6 mm-diameter cores of the representative part of the tumour blocks were implanted in the TMA blocks using Beecher's manual tissue microarrayer (MP06 Beecher Instruments Inc, Sun Prairie, WI, USA).

## 2.3. Immunohistochemistry

LKB1 and 24 other biological markers including ER, progesterone receptor (PgR), Epidermal Growth Factor Receptor (EGFR), Human Epidermal Growth Factor Receptor (HER)-2, HER3, HER4, Breast Cancer Associated gene (BRCA)1&2, p53, Ki67, B-Cell Lymphoma (Bcl)2, Mucin (MUC)1, E-Cadherin, basal (CK5, CK5/6, CK14 and CK17) and luminal (CK7/8, CK18 and CK19) cytokeratins, and Mouse Double Minute (MDM)2 and MDM4 were analysed using indirect IHC by StreptAvidin Biotin Complex and EnVision methods as described [19].

## 2.4. Scoring

Immunohistochemical staining of biological markers was assessed by the percentage of invasive tumour cells stained and by McCarty's immunohistochemical scoring (H-score) (range 0–300) [20]. The LKB1 expression of 30% or more positive cells was considered positive (Figure 2a–d). Previously, studies have shown that the active form of LKB1 is found in the cytoplasm and is associated with other markers and outcome of breast cancer; thus, cytoplasmic expression of LKB1 was considered in our study [21].

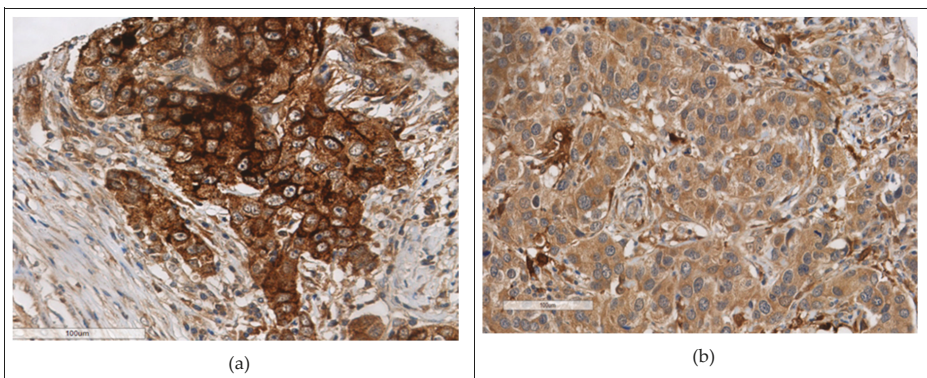
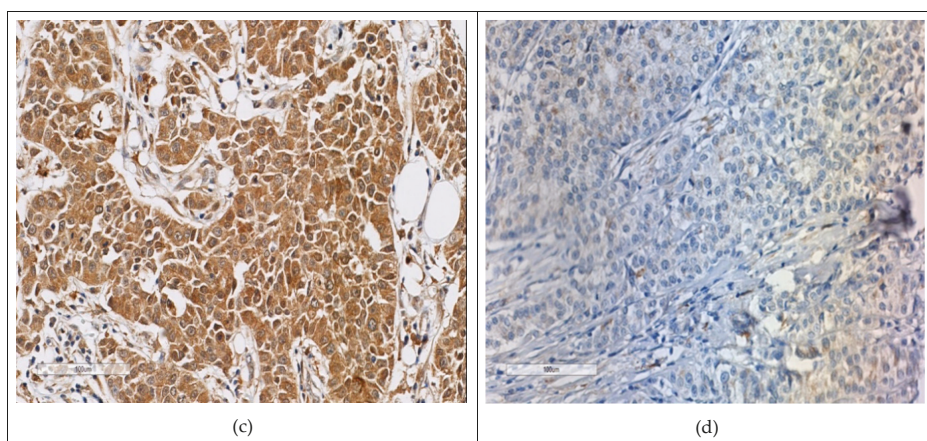


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**Figure 2.** Immunohistochemical expression of LKB1 in Breast cancer cells. (a–c) Positive cytoplasmic expression, (d) LKB1 negative.

### 2.5. Statistical Analysis

X-tile bio-informatics software (Yale University, New Haven, CT, USA) was used to define cut-offs for positivity of the expression of the biological markers based on breast cancer specific survival (BCSS) [22]. The cut-offs for all the biological markers were determined by using the same metric in X-tile. The cut-off values to define positive expression are the same as reported earlier [23]. The Statistical Package for Social Sciences (SPSS, version 16.0, Chicago, IL, USA) was used for data collection and analysis. A Chi-squared test was used for the analysis of the association of the LKB1 with other biomarkers in terms of expression. The clinical outcome was evaluated in terms of BCSS, metastases free survival, local recurrence free survival and regional recurrence free survival, breast cancer specific survival was defined as survival from the date of diagnosis till death from breast cancer; metastases free, local recurrence free and regional recurrence free survivals were calculated from the date of diagnosis till the appearance of the respective recurrences. Survival was analysed by using Kaplan-Meier methods with application of log-rank and generalised Wilcoxon tests, as appropriate. A *p*-value of <0.05 was considered significant.

### 2.6. Ethical Consideration

The study was approved by the Nottingham Research Ethics Committee 2 under ethical approval number C2020313.

## 3. Results

### 3.1. Patients

Of the 575 patients who had TMAs constructed from surgical specimens, 168 cores were lost during the IHC process and 407 cases had LKB1 measured. The median age of the patients was 76 (range 70–91) years, with the majority having had no axillary lymph node involvement (54.2%). Most of the patients ( $n = 296$  (72.7%)) underwent a mastectomy and 46.6% of patients ( $n = 189$ ) received adjuvant endocrine therapy. None of these patients received chemotherapy. The median follow-up was 60 months (longest = 261 months).

### 3.2. Pattern of LKB1 Expression and Association with Other Clinico-Pathological Parameters and Biological Markers

Overall, 78.1% ( $n = 318$ ) of patients showed positive cytoplasmic expression of LKB1 in their tumours.

#### 3.2.1. Pathological Parameters

The expression of LKB1 was significantly associated with high histological grade (among LKB1 positive, grade 1&2 = 44.5%, grade 3 = 55.5%; among LKB1 negative, grade 1&2 = 59.2% and grade 3 = 40.8%,  $p = 0.01$ ). However there was no statistically significant correlation with tumour size (among LKB1 positive, <3 cm = 81.7% and among LKB1 negative, <3 cm = 75.6%,  $p = 0.15$ ) and axillary lymph node status (among LKB1 positive, stage 1&2 = 87.1%, stage 3 = 12.9%, among LKB1 negative, stage 1&2 = 89.5%, stage 3 = 10.5%,  $p = 0.41$ ).

#### 3.2.2. Biological Markers

The positive expression of LKB1 was significantly associated with positive expression of HER2 ( $p = 0.003$ ), Ki67 ( $p = 0.01$ ), VEGF ( $p = 0.002$ ), HER4 ( $p = 0.001$ ), BRCA2 ( $p = 0.01$ ) and MDM2 ( $p < 0.001$ ), and negative expression of CD44 ( $p = 0.03$ ). However, there was no statistically significant correlation with, ER, PgR, p53, basal or luminal cytokeratins, Bcl2, Muc1, EGFR, HER3, MDM4, E-cadherin, and BRCA1.A, as summarised in Table 1.

**Table 1.** Intracellular relationships between LKB1 with biological markers in older women with early operable primary breast cancer.

Biomarker	LKB1 Positive N (%)	LKB1 Negative N (%)	<i>p</i> -Value
ER + ve	211 (69.0)	61 (72.6)	0.30
PgR + ve	168 (55.1)	52 (61.9)	0.16
HER2 + ve	30 (9.6)	2 (2.4)	0.01
Ki67 + ve	114 (35.8)	20 (22.5)	0.01
MUC1 + ve	274 (89.0)	73 (86.9)	0.36
Bcl2 + ve	250 (83.1)	69 (83.1)	0.56
P53 + ve	123 (41.1)	31 (39.7)	0.46
CK5 + ve	104 (33.7)	21 (24.7)	0.07
CK5/6 + ve	128 (45.2)	33 (42.3)	0.37
CK7/8 + ve	301 (97.4)	84 (98.8)	0.38
CK14 + ve	73 (25.8)	15 (18.5)	0.11
CK17 + ve	70 (23.4)	14 (16.7)	0.11
CK18 + ve	286 (97.3)	81 (96.4)	0.45
CK19 + ve	291 (95.4)	79 (96.3)	0.49
EGFR + ve	65 (23.0)	12 (14.8)	0.07
BRCA2 + ve	160 (56.7)	35 (42.7)	0.01
VEGF + ve	234 (88.6)	56 (73.7)	0.002
CD44 + ve	61 (19.7)	25 (29.8)	0.03
MDM2 + ve	296 (96.7)	60 (74.1)	<0.001
E-Cadherin + ve	191 (63.2)	48 (57.1)	0.18
Expression of LKB1 in molecular classes of breast cancer			
Luminal A	79 (76.0)	25 (24.0)	0.09
Luminal B	56 (75.7)	18 (24.3)	
Low ER Luminal	36 (81.8)	8 (18.2)	
All low expression/normal like	13 (76.5)	4 (23.5)	
Basal Like	19 (73.1)	7 (26.9)	
HER2 positive	25 (96.2)	1 (3.8)	

ER: Oestrogen receptor, HER2: Human Epidermal Receptor 2.



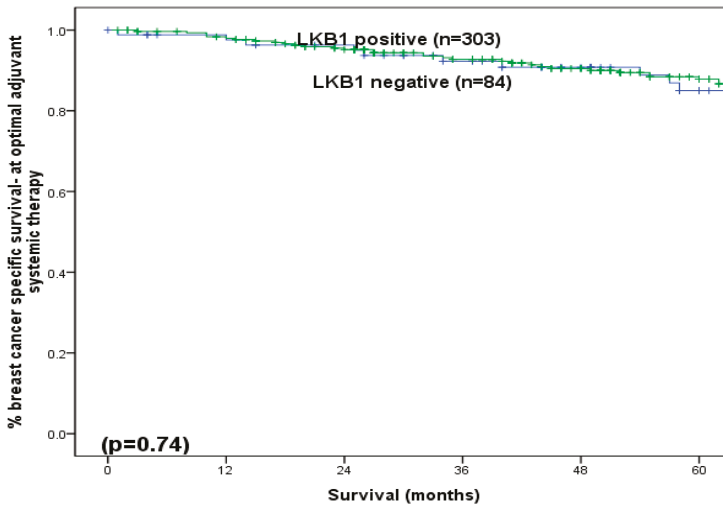
3.3. Correlation with Molecular Classes of Breast Cancer

The expression of LKB1 was correlated with molecular classes (subtypes) of breast cancer found in older women (published previously [23]). The results showed no significant difference in the expression across them (Table 1).

3.4. Association of LKB1 with Clinical Outcome

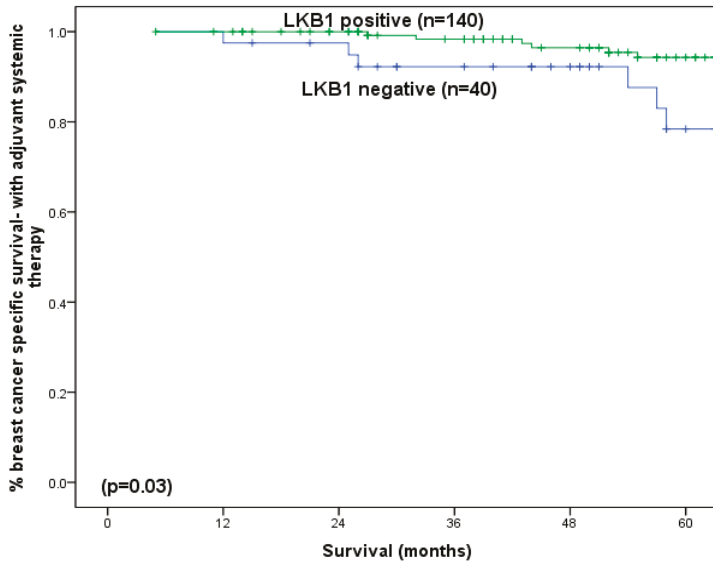
Breast Cancer Specific Survival (BCSS)

At a median follow-up of 60 months (longest 261 months), LKB1 did not show any significant association with BCSS (Figure 3a). However, among those who received adjuvant endocrine therapy ( $n = 267$ ), patients with ER positive disease and positive LKB1 expression (ER+/LKB1+) showed significantly better BCSS (5-year: 93% versus 74%,  $p = 0.03$ ) (Figure 3b). However, in the absence of adjuvant endocrine therapy, LKB1 did not produce any significant impact on survival ( $p = 0.85$ ). For patients with positive LKB1, positive expression of ER ( $p < 0.001$ , Figure 4a), PgR ( $p = 0.001$ , Figure 4b), MUC1 ( $p = 0.003$ , Figure 4c) and Bcl2 ( $p < 0.001$ , Figure 4d) was associated with significantly better BCSS but poorer BCSS in cases of positive expression of HER2 ( $p < 0.001$ , Figure 4e), Ki67 ( $p = 0.01$ , Figure 4f), CK17 ( $p = 0.02$ , Figure 4g) and EGFR ( $p = 0.03$ , Figure 4h).



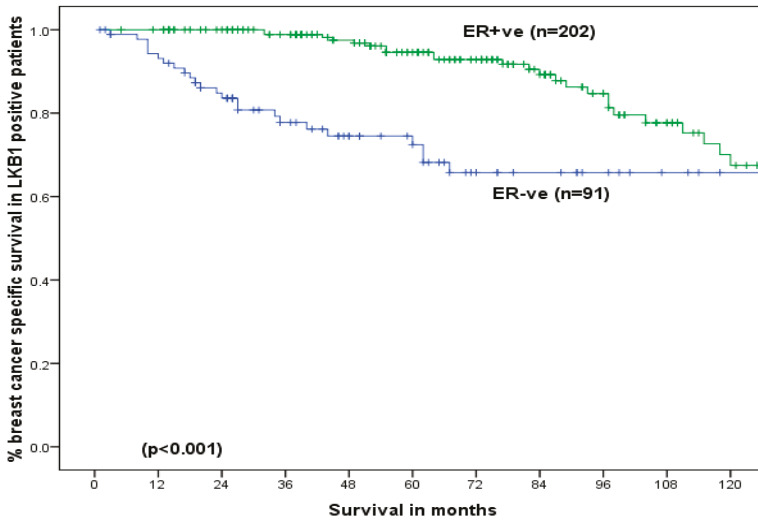
(a)

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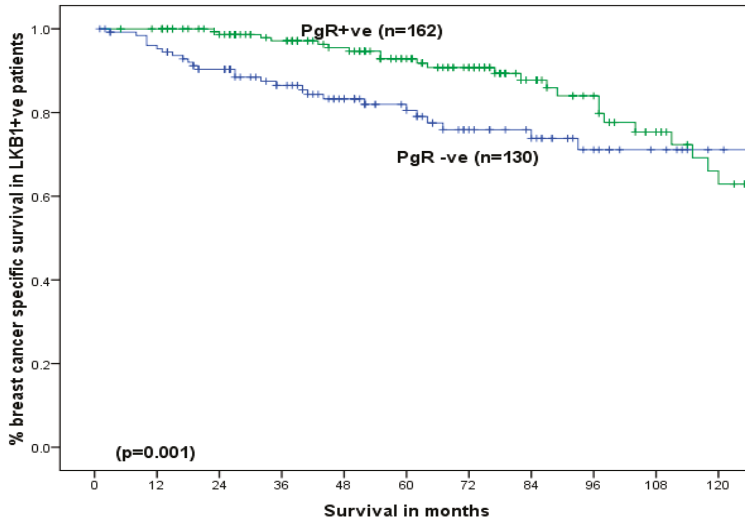
(b)

**Figure 3.** (a) Breast cancer specific survival according to the expression of LKB1 in older women with early operable primary breast cancer (all patients). (b) Breast cancer specific survival according to the expression of LKB1 in older women with early operable primary breast cancer who received adjuvant endocrine therapy.

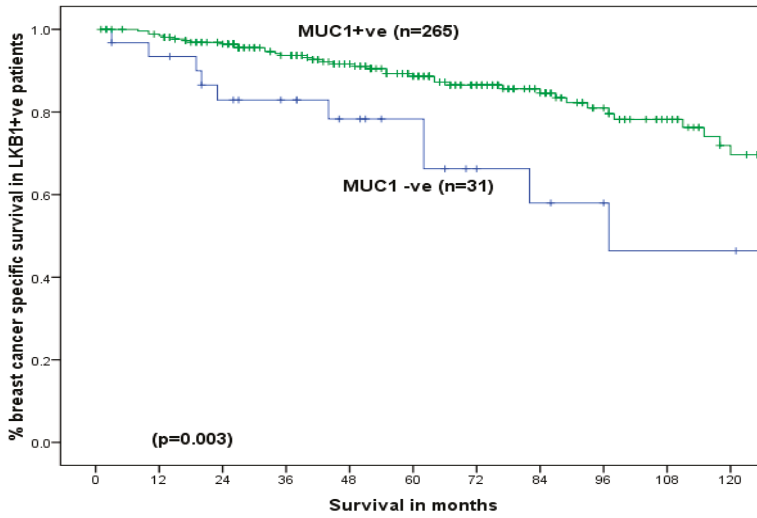


(a)

**Figure 4.** Cont.

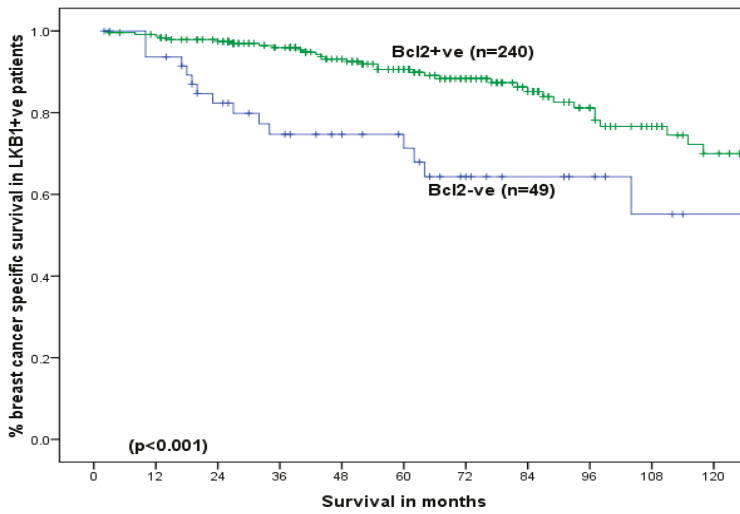


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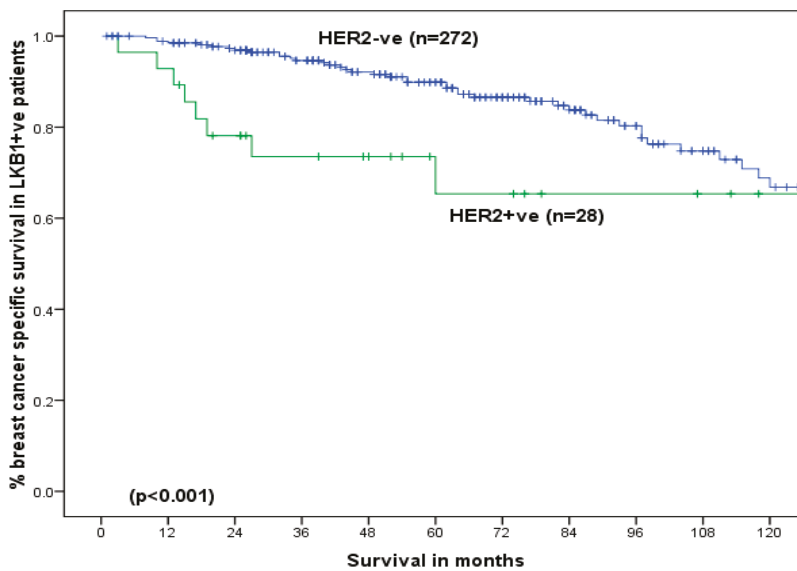


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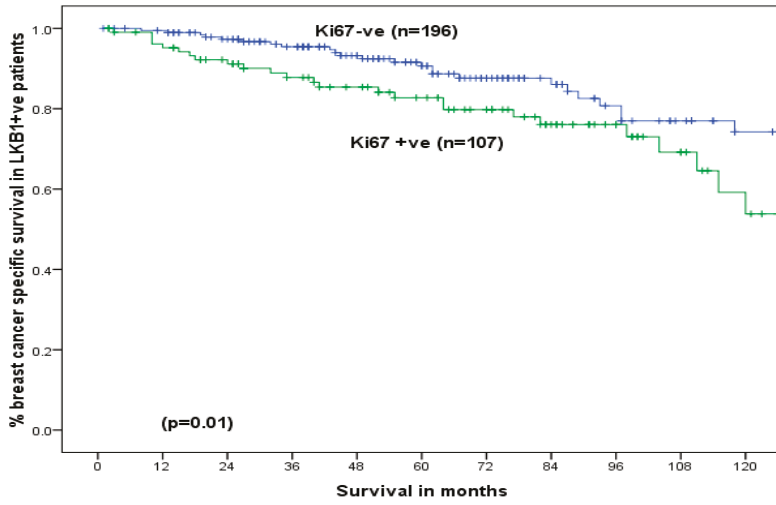


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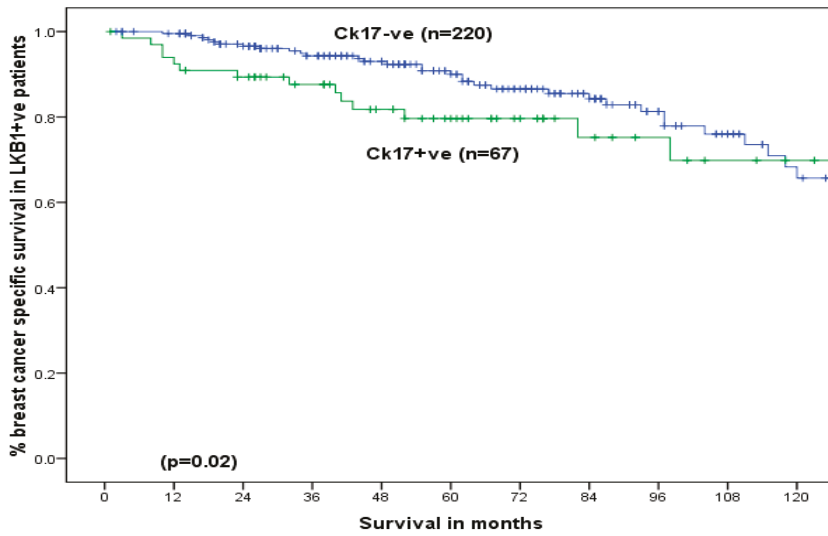


(e)

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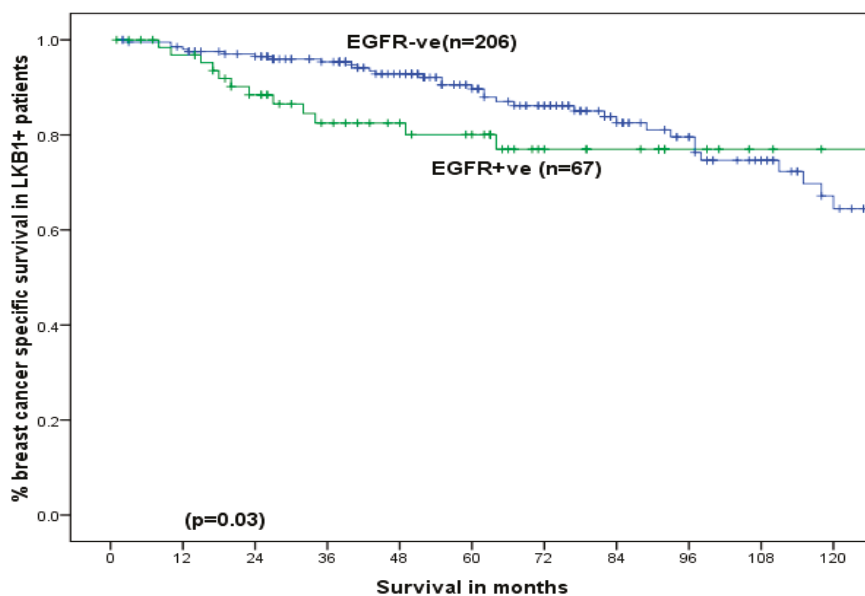


(f)



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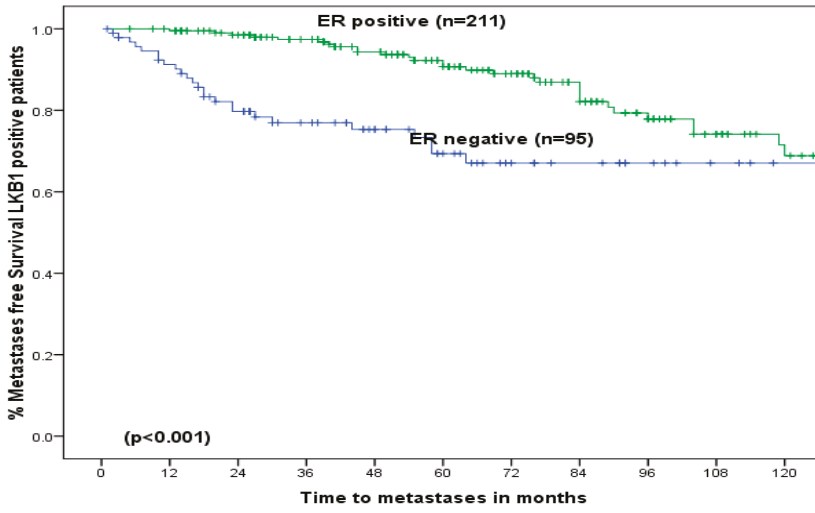


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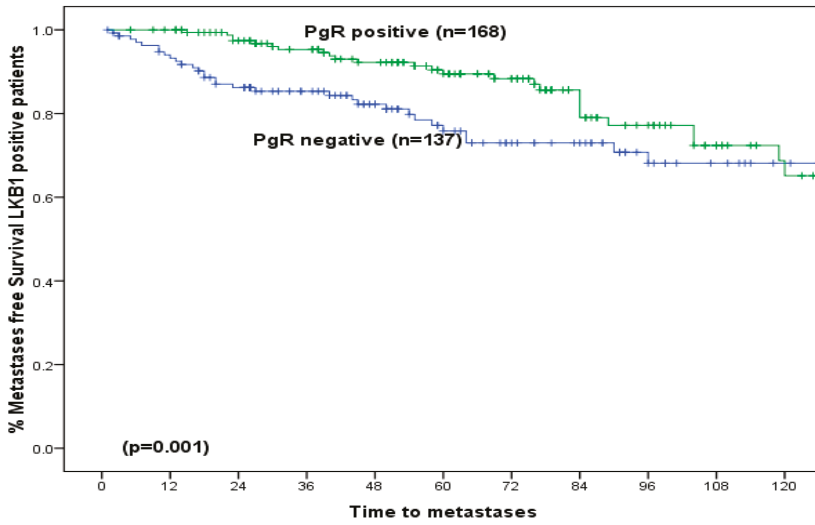
**Figure 4.** Breast cancer specific survival in LKB1 positive early operable primary breast cancer in older women: (a) Oestrogen receptor positive versus oestrogen receptor negative, (b) Progesterone receptor (PgR) positive versus Progesterone Receptor negative, (c) Mucin (MUC)-1 positive versus Mucin 1 negative, (d) B-Cell Lymphoma-2 (Bcl2) positive versus B-Cell Lymphoma 2 negative, (e) Human Epidermal Growth Factor Receptor-2 (HER2) positive versus Human Epidermal Growth Factor Receptor-2 negative, (f) Ki67 positive versus Ki67 negative, (g) Cytokeratin 17 (CK17) positive versus Cytokeratin 17 negative, (h) Epidermal Growth Factor Receptor (EGFR) positive versus Epidermal Growth Factor Receptor negative.

### 3.5. Recurrence Free Survival

There was no significant correlation between metastases-, local recurrence- or regional recurrence-free survival and LKB1 expression ( $p > 0.05$ ). Figure S1a–e). Nevertheless, among patients with positive LKB1, positive expression of ER ( $p < 0.001$ , Figure 5a), PgR ( $p = 0.001$ , Figure 5b), MUC1 ( $p = 0.002$ , Figure 5c), Bcl2 ( $p = 0.004$ , Figure 5d) and negative expression of HER2 were associated with better metastases-free survival ( $p = 0.01$ , Figure 5e). While Ki67 (Figure S2a), CK17 (Figure S2b) and EGFR (Figure S2c) did not show any significant influence on metastases free survival.

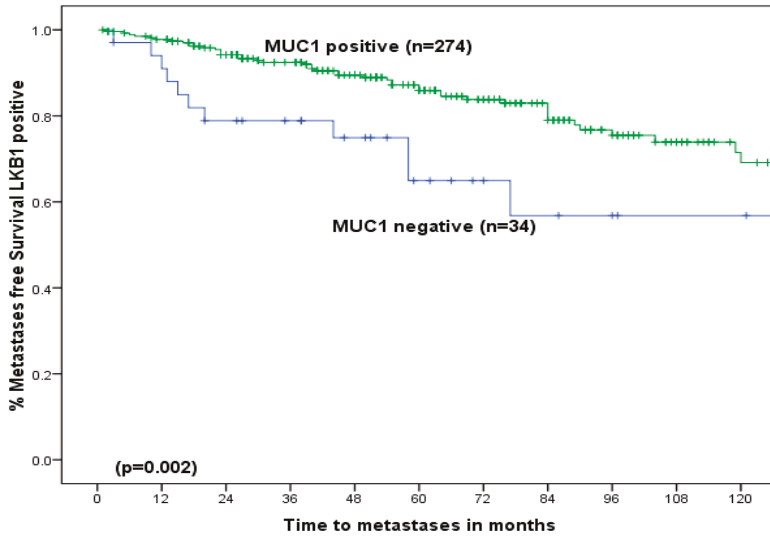


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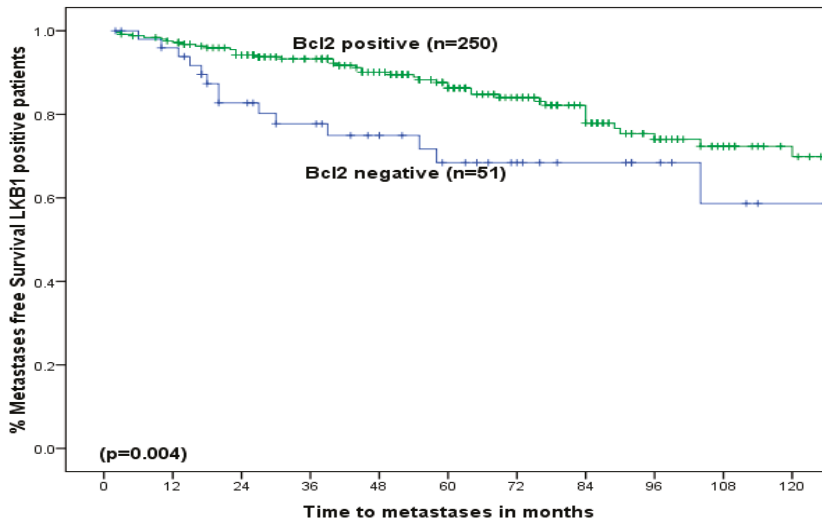


(b)

Figure 5. Cont.



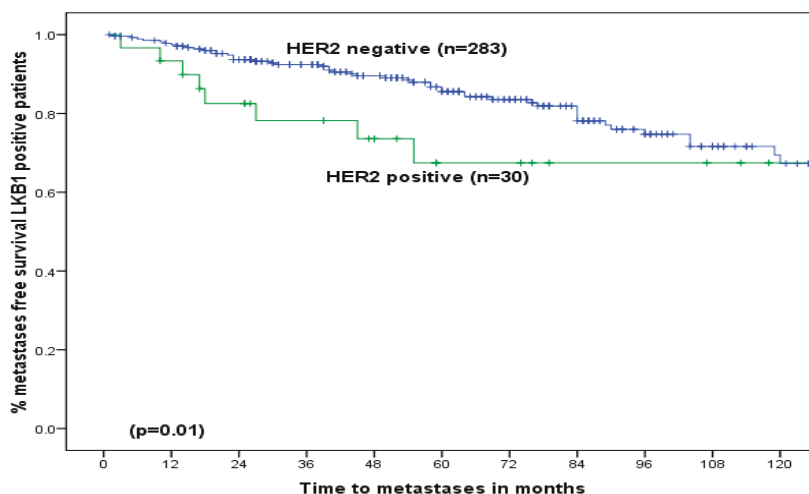
(c)



(d)

Figure 5. Cont.





(e)

**Figure 5.** (a) Metastasis-free survival in LKB1 positive early operable primary breast cancer in older women: ER positive versus ER negative. (b) Metastasis-free survival in LKB1 positive early operable primary breast cancer in older women: PgR positive versus PgR negative. (c) Metastasis-free survival in LKB1 positive early operable primary breast cancer in older women: MUC1 positive versus MUC1 negative. (d) Metastasis-free survival in LKB1 positive early operable primary breast cancer in older women: Bcl2 positive versus Bcl2 negative. (e) Metastasis-free survival in LKB1 positive early operable primary breast cancer in older women: HER2 positive versus HER2 negative.

#### 4. Discussion

Results of the analysis showed that cytosolic expression of LKB1 was associated with high-grade tumours and positive expression of HER2, Ki67 and VEGF. Within the population with LKB1 positive tumours, BCSS was significantly better in patients who received adjuvant endocrine therapy.

The expression of LKB1 in tumours cells was previously reported as 71%, which is slightly lower than the expression reported in our population (78%) [24]. This could be explained by the age of the population, as our study included patients 70 years or older, while the previously reported data included four groups with median ages of 48, 54, 61 and 62 years [24]. In our study, the association between LKB1 positivity and the factors mentioned above suggests its role as a possible poor prognostic indicator. The association of LKB1 expression with high histological grade suggests a poor prognostic indication as reported previously, where IHC was used for the detection of LKB1 protein expression [24]. While in vitro studies reported a negative correlation between both LKB1 mutation and grade and VEGF and a positive correlation with hormone receptor expression [1,3,12,25], these studies analysed LKB1 using Western blot and RT-PCR [1,3,11]. A recently presented study suggested that the nuclear location of LKB1 was associated with better survival while cytosolic expression is an indicator of poor prognosis [25]. Another study analysed both nuclear and cytoplasmic expression ( $n = 32$ ) on IHC in early operable primary breast cancer with ER and PgR positive expression [26]. High cytoplasmic expression was seen in 62.5% of cases, which was not associated with age, ER, PgR and HER2, but was shown to be significantly associated with smaller tumour size [26]. In this study, the nuclear expression was also associated with smaller tumours and better survival. A high LKB1 gene expression has been reported to be linked to a high ER expression [24]. The expression of LKB1 has shown its association with CD44, which is a marker of stemness in breast cancer stem cells. Previously, the available literature showed LKB1 as a factor for maintenance of stem cells in haemopoietic cells in a

mouse model study, where animals with LKB1 deletion had progressive pancytopenia and reduction in haemopoietic cell proliferation [27–29]. In this study, LKB1 has also shown its significant association with VEGF, probably linked to tumour growth and invasion. A recent study has demonstrated the influence of LKB1 on tumour invasion and metastases in colorectal cancer and suggested it to be a potential therapeutic marker [30].

The expression of LKB1 among molecular classes or subtypes has not been reported in the literature. Though it did not show any remarkable findings in our data analyses, its relatively high expression in the HER2 over-expressing class may be linked to some relationship with growth factor pathways. As it was previously reported, it may be a potential predictor of overall survival among HER2 positive breast cancer patients [24].

The prognostic significance of LKB1 was clinically demonstrated in the group of patients who received adjuvant endocrine therapy. This is an interesting finding, which is in line with the data previously reported, which suggested that within the stress environment, 17 $\beta$  oestradiol stimulates the AMPK pathway. The same study has also suggested that LKB1 is required for the ER mediated activation of AMPK [31,32]. The tumour analysis of the patients included in the TAMRAD randomized GINECO trial (the trial compared tamoxifen versus tamoxifen plus everolimus in ER+ metastatic breast cancers). It reported that the patients with a low cytoplasmic expression of LKB1 demonstrated a 67% reduction in the risk of disease progression with everolimus (a mammalian target of rapamycin (mTOR) inhibitor) plus tamoxifen, when compared to the tamoxifen only arm [33]. On the other hand, high cytoplasmic expression was associated with less benefit with everolimus therapy, consistent with our results, suggesting the predictive role of LKB1 in patients with ER positive tumours.

In addition, LKB1 has been previously reported to inhibit the aromatase enzyme via the AMP-activated protein kinase, thus supporting its potential role in the prediction of the response to primary endocrine therapy in older women [7,10,26].

The study has limitation of not including the comparison with the younger women and not studying the gene expression and mutations in older women. Precisely defining an expression cut-off based on IHC remains a challenge as this could vary between laboratories.

## 5. Conclusions

The available literature reported on LKB1 are primarily based on in vitro and in vivo studies using Western blot or PCR techniques. Our work describes a novel study which analysed the relationship between LKB1 and other biomarkers and clinical outcome in a large series of older patients with breast cancer by using IHC. Given the availability of IHC in clinical practice, this study possesses translational significance. The study has demonstrated significant correlations between LKB1 and biological markers of established significance. It has also shown its significant influence on the clinical outcome (notably BCSS) in older women receiving adjuvant endocrine therapy. Further studies are required to investigate and compare the expression of LKB1 in and with the younger patients and to delineate the precise role of LKB1 as a potential therapeutic target in breast cancer management. Studies are also required to understand the detailed genetics of LKB1.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/11/2/149/s1>, Figure S1: (a) Metastasis-free survival according to the expression of LKB1 in older women with early operable primary breast cancer (all patients). (b) Local recurrence-free survival according to the expression of LKB1 in older women with early operable primary breast cancer (all patients). (c) Regional recurrence free survival according to the expression of LKB1 in older women with early operable primary breast cancer (all patients). (d) Metastasis-free survival according to the expression of LKB1 in older women with early operable primary breast cancer who received adjuvant endocrine therapy, Figure S2. (a) Metastases free survival in LKB1 positive early operable primary breast cancer in older women: Ki67 positive versus Ki67 negative. (b) Metastasis-free survival in LKB1 positive early operable primary breast cancer in older women: CK17 positive versus CK17 negative. (c) Metastasis-free survival in LKB1 positive early operable primary breast cancer in older women: EGFR positive versus EGFR negative.

**Author Contributions:** B.M.S. designed study, collected data, analysed data and wrote manuscript; A.R.G. supervised study, analysed data, written manuscript; D.A.L.M. supervised study, analysed data and wrote

manuscript; I.O.E.: supervised study, analysed data and wrote manuscript; K.L.C. conceived idea, designed study, collected data, analysed data and wrote manuscript.

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**Conflicts of Interest:** Authors declare no conflict of interest.

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Article

# Cytoplasmic Cyclin E Is an Independent Marker of Aggressive Tumor Biology and Breast Cancer-Specific Mortality in Women over 70 Years of Age

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**Abstract:** Multi-cohort analysis demonstrated that cytoplasmic cyclin E expression in primary breast tumors predicts aggressive disease. However, compared to their younger counterparts, older patients have favorable tumor biology and are less likely to die of breast cancer. Biomarkers therefore require interpretation in this specific context. Here, we assess data on cytoplasmic cyclin E from a UK cohort of older women alongside a panel of >20 biomarkers. Between 1973 and 2010, 813 women  $\geq 70$  years of age underwent initial surgery for early breast cancer, from which a tissue microarray was constructed ( $n = 517$ ). Biomarker expression was assessed by immunohistochemistry. Multivariate analysis of breast cancer-specific survival was performed using Cox's proportional hazards. We found that cytoplasmic cyclin E was the only biological factor independently predictive of breast cancer-specific survival in this cohort of older women (hazard ratio (HR) = 6.23, 95% confidence interval (CI) = 1.93–20.14;  $p = 0.002$ ). At ten years, 42% of older patients with cytoplasmic cyclin E-positive tumors had died of breast cancer versus 8% of negative cases ( $p < 0.0005$ ). We conclude that cytoplasmic cyclin E is an exquisite marker of aggressive tumor biology in older women. Patients with cytoplasmic cyclin E-negative tumors are unlikely to die of breast cancer. These data have the potential to influence treatment strategy in older patients.

**Keywords:** breast cancer; cyclin E; older patients; biomarker; tumor biology; prognosis; survival

## 1. Introduction

The prognosis for patients with primary breast cancer is a function of disease extent at presentation and tumor biology. Time-dependent factors, including metastatic involvement of draining axillary lymph nodes (stage) and the size of the primary tumor, indicate disease extent, while the degree of differentiation (grade) is used as a surrogate for biological aggressiveness. These histopathology-assessed features are classically combined into the Nottingham Prognostic Index (NPI) [1].

Due to comorbidities, older patients with primary breast cancer are more likely to die of non-breast cancer-related causes when compared to their younger counterparts [2,3]. Furthermore, tumors in older patients have distinct biological characteristics that are linked to favorable outcome, such as a higher proportion and degree of hormone receptor (estrogen receptor (ER) and/or progesterone receptor (PR)) positivity, and low histological grade and Ki67 proliferative index [4,5]. Biomarkers that may influence treatment strategy therefore require interpretation in the specific clinical and biological context of older women.

Recent developments in risk stratification, such as tumor gene expression profiling (e.g., Oncotype Dx and MammaPrint), have focused on the biology of the primary tumor, based on the premise that this reflects the biology of presumed micrometastatic deposits [6,7]. Adjuvant therapy is then selected according to risk category with the aim of preventing these deposits from developing into clinically detectable metastases, thus avoiding disease relapse.

We have previously shown that tumor biology in older (>70 years) versus younger primary breast cancer cohorts can be distinguished by immunohistochemistry (IHC) using a panel of biomarkers, including ER, PR, HER2, Ki67, Bcl2, BRCA1, BRCA2, E-Cadherin, EGFR, LKB1, MDM2, MDM4, MUC1, p53, VEGF and cytokeratin markers of luminal and basal disease [4]. Differential expression of age-associated biomarkers clustered tumors from older patients into six groups with distinct clinical outcomes.

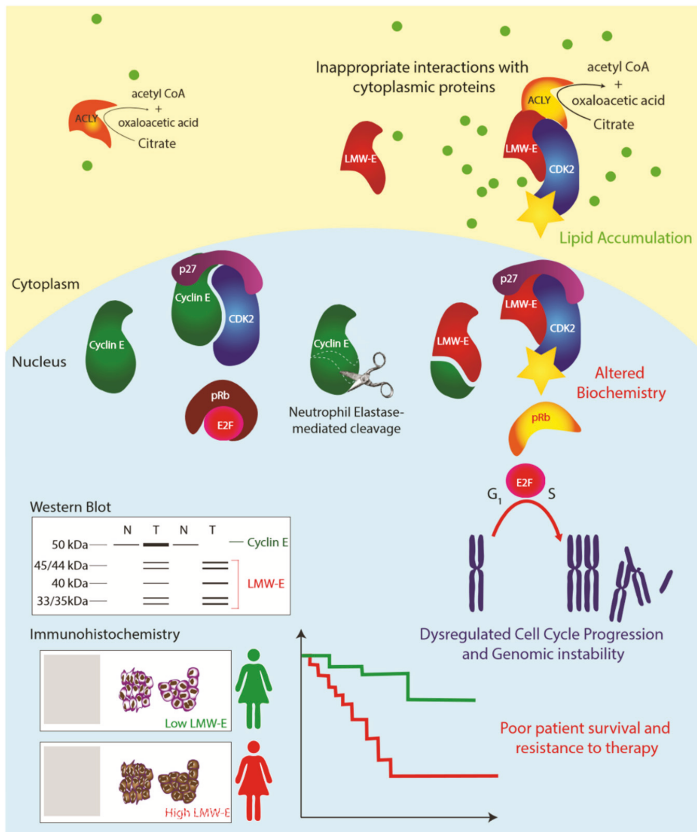
The current study focuses on the role of G1/S-specific cyclin-E1 (cyclin E) as a biological marker of aggressive disease, and on its prognostic significance in the context of older women with primary breast cancer.

In normal dividing cells, cyclin E promotes the transition from G1 to S phase by activating cyclin-dependent kinase 2 (CDK2) [8]. In breast cancer cells, tumor-specific proteolytic processing of cyclin E generates hyperactive low molecular weight (LMW) isoforms [9]. In contrast to full length cyclin E, for which prognostic data have been equivocal, LMW isoforms are highly prognostic in primary breast cancer patients [10–12]. As they lack a portion of the amino-terminus containing a nuclear localization sequence, LMW isoforms of cyclin E preferentially accumulate in the cytoplasm where they evade nuclear FBW-7 ubiquitin ligase that would otherwise increase their turnover [13].

In previous work using an antibody named C-19 that interacts with the carboxyl-terminus of cyclin E (present in both full length and LMW isoforms of cyclin E), we showed that transgenic mouse models with mammary gland expression of LMW cyclin E developed mammary tumors positive only for cytoplasmic cyclin E [14]. Furthermore, cytoplasmic cyclin E bound to its catalytic subunit, CDK2, in the cytoplasm of tumor cells [14]. The biological functions of cytoplasmic, LMW cyclin E are summarized in Figure 1.

These critical findings on the biological functions of LMW cyclin E paved the way for a pivotal study on the use of cytoplasmic expression of cyclin E (c-cyclin E) by IHC to predict recurrence in patients with primary breast cancer [15]. Combined analysis of 2494 tumors from four cohorts of patients (from the UK and the USA, covering all age groups) presenting with primary breast cancer demonstrated that c-cyclin E staining is highly prognostic. These data included the current UK cohort of older women (median age 76, versus 53–62 years in all cohorts,  $p < 0.0001$ ).

We now present data on c-cyclin E exclusively from the Nottingham cohort. This paper is distinct from the multi-cohort study as it focuses only on the older population and assesses the role of c-cyclin E against a large panel of more than 20 disease markers. Findings are interpreted in the specific biological and clinical context of primary breast cancer in older women, and the implications for risk stratification and treatment decision-making in older patients are discussed.



**Figure 1.** Model: Low molecular weight cyclin E (LMW-E) isoforms are generated by neutrophil elastase-mediated proteolytic cleavage, removing the N-terminal nuclear localization signal. LMW-E isoforms accumulate in the cytoplasm where they inappropriately interact with cytoplasmic proteins, such as ACYL. The altered biochemistry of LMW-E results in hyperactivation of CDK2, resistance to endogenous CDK-inhibitors (p27CIP1 and p27KIP1) and altered substrate interactions, which results in enhanced cell cycle progression, genomic instability and other pro-tumorigenic features. Evaluation of LMW-E by Western blot (protein size) or immunohistochemistry (cytoplasmic localization) is prognostic of poor prognosis and predicts failure of standard treatment modalities.

**2. Results**

Patient clinicopathological characteristics are summarized in Table 1. Median follow-up was 6.3 years (95% CI, 6.1–7.1 years).

**Table 1.** Summary of patient characteristics (n = 517).

Variable	Group	n	%
age	70 to <75 yrs	206	39.8%
	75 to <80 yrs	202	39.1%
	80 to <85 yrs	83	16.1%
	≥85 yrs	26	5.0%
tumor size	<20 mm	153	31.7%
	≥20 mm	329	68.3%



Table 1. Cont.

Variable	Group	n	%
stage	1	183	57.5%
	2	98	30.8%
	3	37	11.6%
grade	1	51	12.0%
	2	169	39.8%
	3	205	48.2%
ER status	negative	143	29.9%
	positive	335	70.1%
HER2 status	negative	453	91.7%
	positive	41	8.3%
Metastasis <sup>1</sup>	negative	430	83.2%
	positive	87	16.8%

<sup>1</sup> at last follow-up.

### 2.1. Expression of Cytoplasmic Cyclin E Associates with High-Grade, ER-/PR- Breast Tumors with High Ki67 Proliferative Index

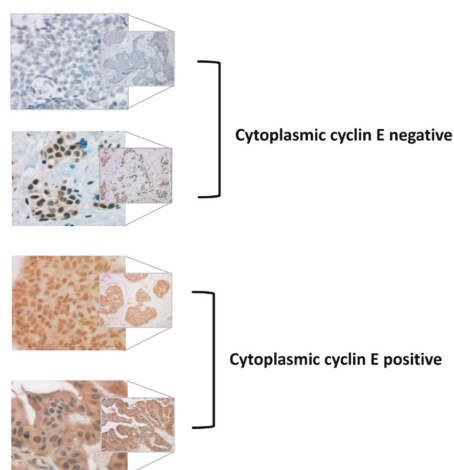
We examined the expression of c-cyclin E and a panel of biomarkers in breast tumors by IHC. Average concordance between pathologists was 93% for the Nottingham cohort (see Table 2).

**Table 2.** Concordance between two independent pathologists for the Nottingham cohort ( $n = 516$ ).

		Pathologist B		Total
		c-cyclin E+	c-cyclin E−	
Pathologist A	c-cyclin E+	199	11	210
	c-cyclin E−	11	295	306
				516

Representative IHC staining for c-cyclin E positive and negative cases is presented in Figure 2.

To assess the potential clinical relevance of c-cyclin E, we first compared c-cyclin E expression with clinicopathological features at initial diagnosis (age, tumor size, stage and grade) and markers of disease biology in current clinical use, including ER, PR, HER2 and Ki67 status.



**Figure 2.** Representative images of positive and negative staining for cytoplasmic cyclin E using antibody C-19.

There was a strong association between c-cyclin E expression and higher grade ( $p < 0.0005$ , see Table 3). In contrast, there was no association between c-cyclin E and patient age, tumor size or stage. Cytoplasmic expression of cyclin E was significantly associated with negative ER and PR status ( $p = 0.002$  and  $p = 0.012$ , respectively) and high Ki67 proliferative index ( $p = 0.047$ ) (Table 3). No significant association was found between c-cyclin E and HER2 status.

**Table 3.** Association between tumor c-cyclin E status and clinicopathological factors.

Variable		c-Cyclin E−		c-Cyclin E+		<i>p</i>
age	70 to <80 yrs	170	(41.7%)	238	(58.3%)	0.743
	≥80 yrs	43	(39.4%)	66	(60.6%)	
size	<20 mm	64	(41.8%)	89	(58.2%)	0.921
	≥20 mm	135	(41.0%)	194	(59.0%)	
stage	1	83	(45.4%)	100	(54.6%)	0.350
	2	42	(42.9%)	56	(57.1%)	
	3	12	(32.4%)	25	(67.6%)	
grade	1	33	(64.7%)	18	(35.3%)	<0.0005 <sup>1</sup>
	2	87	(51.5%)	82	(48.5%)	
	3	64	(31.2%)	141	(68.8%)	
ER	negative	43	(30.1%)	100	(69.9%)	0.002 <sup>1</sup>
	positive	151	(45.1%)	184	(54.9%)	
PR	negative	74	(34.6%)	140	(65.4%)	0.012 <sup>1</sup>
	positive	122	(46.2%)	142	(53.8%)	
HER2	negative	187	(41.3%)	266	(58.7%)	0.869
	positive	16	(39.0%)	25	(61.0%)	
Ki67	negative	152	(44.3%)	191	(55.7%)	0.047 <sup>1</sup>
	positive	61	(35.1%)	113	(64.9%)	

<sup>1</sup> Statistical significance  $p < 0.05$ , by  $\chi^2$  test

Comparison of c-cyclin E status with other biomarkers revealed a positive association with VEGF ( $p = 0.041$ ), and no other significant association.

## 2.2. Cytoplasmic Cyclin E Expression Is Enriched in Basal Tumors

We next assessed the association between tumor c-cyclin E expression and cellular phenotype as indicated by the expression of cytokeratin markers in the IHC protein panel.

Cytoplasmic cyclin E expression was associated with markers of basal disease (see Table 4). Basal cytokeratin markers significantly associated with c-cyclin E included CK5 and CK17 ( $p = 0.001$  and  $p = 0.036$ , respectively). In contrast, there was no association between c-cyclin E and the luminal marker CK18.

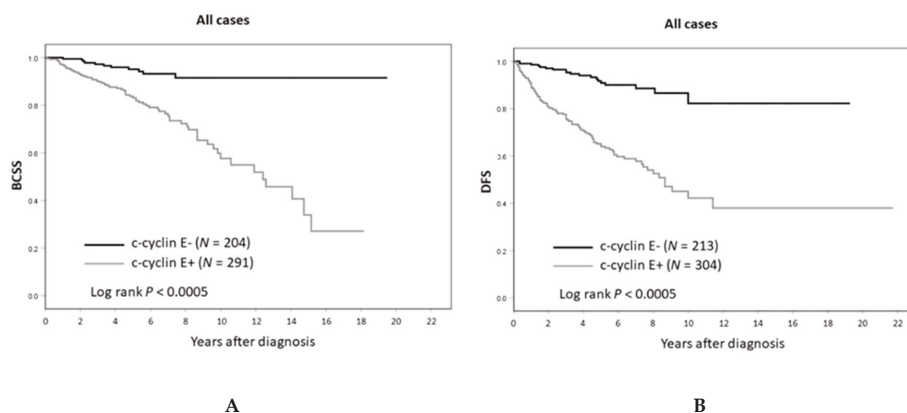
**Table 4.** Association between tumor c-cyclin E status and clinicopathological factors.

Variable		c-Cyclin E−		c-Cyclin E+		<i>p</i>
CK5	negative	152	(45.5%)	182	(54.5%)	0.001 <sup>1</sup>
	positive	43	(29.5%)	103	(70.5%)	
CK5/6	negative	101	(43.3%)	132	(56.7%)	0.284
	positive	78	(37.9%)	128	(62.1%)	
CK14	negative	143	(43.7%)	184	(56.3%)	0.074
	positive	37	(33.9%)	72	(66.1%)	
CK17	negative	160	(43.1%)	211	(56.9%)	0.036 <sup>1</sup>
	positive	30	(31.3%)	66	(68.8%)	
CK18	negative	3	(21.4%)	11	(78.6%)	0.170
	positive	187	(41.9%)	259	(58.1%)	

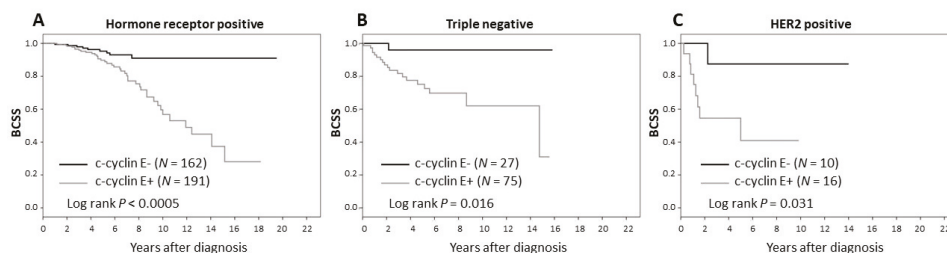
<sup>1</sup> statistical significance  $p < 0.05$ , by  $\chi^2$  test. CK5, CK5/6 (antibody to both CK5 and CK6), CK14 and CK17 are basal markers; CK18 is a luminal marker.

### 2.3. Survival Analysis

Kaplan–Meier plots of breast cancer-specific survival (BCSS) and disease-free survival (DFS) as a function of c-cyclin E status are shown in Figure 3. Lack of c-cyclin E was associated with good prognosis in the patient cohort (BCSS and DFS both  $p < 0.0005$  by logrank test). This was observed for luminal A/B (ER+ and/or PR+), HER2+ and triple negative breast cancer subtypes (see Figure 4).



**Figure 3.** (A) Breast cancer-specific and (B) disease-free survival by cytoplasmic cyclin E status.



**Figure 4.** Breast cancer-specific survival by subtype: (A) hormone receptor (ER and/or PR) positive, (B) triple negative, (C) HER2 positive.

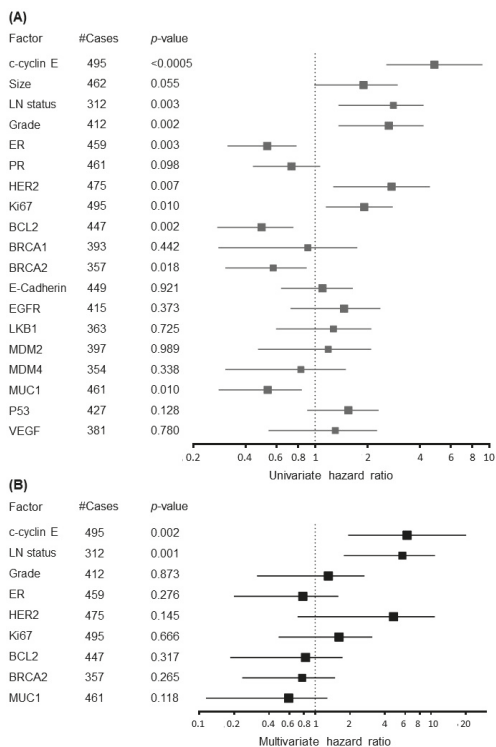
Survival analysis of c-cyclin E alongside the full panel of biomarkers was performed using data up to last follow-up. Due to the low proportion of low-grade tumors (grade 1, 12%), these were combined with intermediate-grade tumors (grade 2, 40%) and used as a statistical reference for comparison with high-grade tumors (grade 3, 48%). Multivariate analysis was performed on all clinicopathological factors and biomarkers significantly associated with BCSS in univariate testing.

Cytoplasmic expression of cyclin E was the only independent biomarker of BCSS and had a strong association in multivariate analysis (HR = 6.23, 95% CI 1.93–20.14;  $p = 0.002$ ) (Figure 5). The only clinicopathological factor predictive of BCSS in the multivariate analysis was axillary nodal status (HR = 4.38, 95% CI 1.77–10.84;  $p = 0.001$ ).

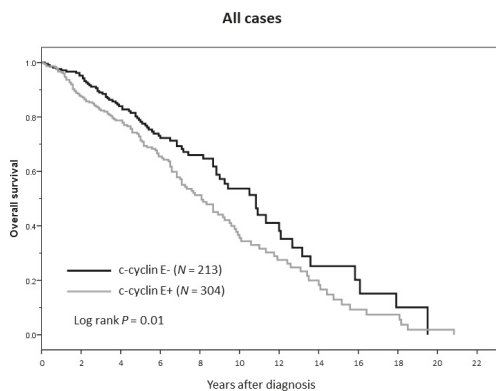
For the whole cohort of 517 patients, there was a strong positive association between c-cyclin E positivity and breast cancer-specific mortality at 5 years of follow-up ( $p < 0.0005$ ). Multivariate analysis demonstrates that c-cyclin E positivity is the strongest predictor of 5-year BCSS in this cohort (HR = 9.12, 95% CI 2.22–37.40;  $p = 0.002$ )—outperforming lymph node status (HR=4.49, 95% CI 1.66–12.15;  $p = 0.003$ ) and all other biological disease markers.

At ten years of follow-up, BCSS for patients with c-cyclin E-negative tumors was 92% versus 58% for those with c-cyclin E-positive tumors (HR = 6.23, 95% CI 1.92–20.14;  $p = 0.002$  in multivariate

analysis). At completion of follow-up, the absolute difference in median overall survival (OS) by c-cyclin E status (positive versus negative) was 33 months (130 versus 97 months,  $p = 0.01$  by logrank, Mantel–Cox) (see Figure 6).



**Figure 5.** (A) Univariate and (B) multivariate analysis of c-cyclin E with clinicopathological and age-associated biomarkers.

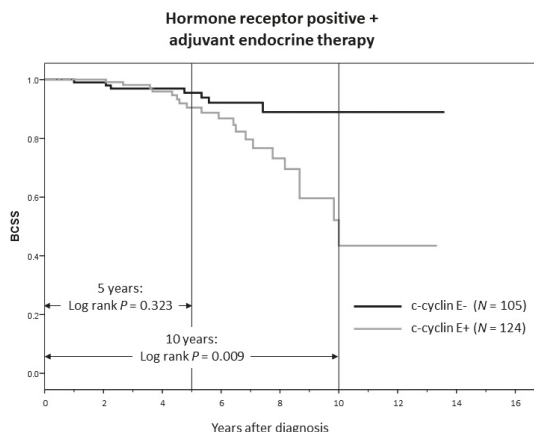


**Figure 6.** Overall survival (all cases) by cytoplasmic cyclin E status.

## 2.4. Prognostic Value of Cytoplasmic Cyclin E in Patients Treated with Endocrine Therapy

Patients with ER+ disease received up to 5 years of adjuvant endocrine therapy according to risk stratification by NPI (e.g.,  $NPI > 2.4$  or  $3.4$ , according to best practice guidelines at the time of treatment). We next explored the prognostic value of c-cyclin E and its relationship to adjuvant endocrine therapy in patients with ER+ tumors.

In contrast to the whole cohort (where c-cyclin E was the strongest predictor of 5-year BCSS), in patients who received up to 5 years of adjuvant endocrine therapy ( $n = 229$ ), c-cyclin E was not associated with 5-year BCSS ( $p = 0.323$ ). After cessation of adjuvant endocrine therapy, c-cyclin E reached prognostic significance (positive association with breast cancer-specific mortality) at 10 years of follow-up ( $p = 0.009$ ) (see Figure 7).



**Figure 7.** Adjuvant endocrine therapy negates the poor prognostic effect of c-cyclin E positivity, but only for as long as it is given (up to 5 years).

## 3. Discussion

In this study of 517 older women with primary breast cancer, c-cyclin E is the only independent biological marker of disease outcome. In terms of prognostic value, c-cyclin E status outperforms all standard clinical and age-associated biomarkers tested in this study.

For the older population, who more often present with complex comorbidities and psychosocial factors, considerations regarding competing causes of death are paramount in clinical decision-making [16]. The current study presents BCSS as a surrogate measure for the impact of tumor biology on patient mortality. Deaths due to non-breast cancer causes are excluded from the analysis. Unlike non-modifiable and time-dependent clinicopathological disease factors, such as tumor size and stage, those factors pertaining to tumor biology can be modified using systemic therapy.

Data from the current study supplement findings from an international study of four cohorts (including the Nottingham cohort) and add clinical context in older women [16]. In the combined analysis, freedom from recurrence (FFR), defined as the time between the date of diagnosis and the date of first locoregional or distant recurrence, was the primary endpoint. Unlike BCSS and DFS, FFR does not include death as an event, regardless of the cause of death.

In two of the other three cohorts in the combined analysis (MD Anderson (MDA) and National Cancer Institute (NCI)), c-cyclin E expression had the strongest effect on FFR. In the Nottingham cohort, c-cyclin E outperformed standard clinical biomarkers in terms of both FFR and BCSS.

Combined analysis of the two largest US clinical cohorts contributing to the multi-cohort study has also been reported (MDA and NCI) [14]. Data from the Nottingham cohort are consistent with the NCI and MDA cohorts. For example, there was 60% positive c-cyclin E staining in combined NCI/MDA

analysis, compared to 59% in the Nottingham cohort. This demonstrates good reproducibility of IHC measurement of cytoplasmic expression of cyclin E—an important factor when considering the potential application of c-cyclin E status as a clinical biomarker.

In line with the Nottingham cohort, expression of c-cyclin E in the combined analysis of the NCI and MDA cohorts was associated with tumor markers, such as grade, ER and PR, and was associated with poor patient outcome. However, in the NCI/MDA analysis, c-cyclin E was only an independent marker of recurrence-free and overall survival when combined with the downstream kinase, cyclin-dependent kinase 2. In the NCI/MDA analysis, histological grade was also an independent prognostic factor.

Analysis of the Nottingham cohort of older women, in contrast, found that c-cyclin E was the only independent prognostic biomarker, outperforming even tumor grade in terms of prognostic value. Although c-cyclin E was not associated with age in any of the cohorts, overall findings from the older Nottingham cohort suggest a greater biological effect of high c-cyclin E expression in the older population. Breast cancer biology in the older population is considered more indolent, with less aggressive phenotypic features and enrichment for ER expression. This suggests that c-cyclin E has greater potential clinical utility in the older population and is better able to distinguish more aggressive tumors that are likely to lead to death from breast cancer.

Findings from the current study support c-cyclin E as a robust and reproducible biomarker of an aggressive disease course. The findings suggest that clinical outcomes of older women with primary breast cancer can be predicted from the biology of their tumor using c-cyclin E alone. Older patients with c-cyclin E-negative tumors are unlikely to die of their breast cancer. At ten years, only 8 patients out of 100 will die of their breast cancer if their tumor is c-cyclin E-negative. For many older patients, this finding will directly impact clinical decision-making.

Alongside existing clinical decision-making tools such as comprehensive geriatric assessment scales and biomarkers of treatment response such as ER expression, there is clear potential to utilize tumor c-cyclin E expression in the clinic. For example, c-cyclin E positivity may indicate a requirement for an aggressive initial treatment strategy, in terms of surgical management of the primary tumor and axillary lymph nodes, and the need for adjuvant therapy.

Although the clinical setting of the current study is patients selected to undergo initial surgery, who are more likely to have ER-negative tumors, it may be possible to extrapolate its findings on the value of c-cyclin E as a marker of aggressive disease biology to other clinical scenarios.

For example, ER/PR+ primary tumors that do not express c-cyclin E may be adequately treated by primary endocrine therapy. Alongside comprehensive geriatric assessment, c-cyclin E may serve as a biomarker in this context for patients who, because of comorbidity, psychosocial factors or individual choice, would prefer not to undergo initial surgery. Given the high proportion of older patients already receiving primary endocrine therapy (up to 40% of UK patients in the previous decade), there is a compelling case to investigate this hypothesis in prospective clinical trials [17].

In adjuvant therapy decision-making, c-cyclin E could supplement or replace gene expression predictors of chemotherapy response (e.g., Oncotype Dx and MammaPrint). Additionally, as cyclin-dependent kinase 2-targeting treatments, e.g., dinaciclib, emerge from early phase clinical trials, there may be a role for these drugs in older patients in preference to chemotherapy [18,19].

It is reported that c-cyclin E mediates resistance to endocrine therapy with aromatase inhibitors (AIs) in breast cancer [20]. This study suggests that c-cyclin E may identify ER/PR+ tumors that are unresponsive to AIs, which do not induce a cytostatic effect.

For patients with ER+ disease, evidence from the current study suggests that adjuvant endocrine therapy negates the poor prognostic effect of c-cyclin E positivity. This implies that extending adjuvant therapy beyond 5 years would improve long-term outcomes and would be a priority for patients with c-cyclin E-positive tumors.

#### 4. Materials and Methods

Over a 37-year period (1973–2010), 1758 older ( $\geq 70$  years) women with stage I–II primary breast cancer were managed in a dedicated facility in Nottingham, as previously described [21]. Of these patients, 813 underwent primary surgery.

Good quality formalin-fixed, paraffin embedded tissue from 575 tumors was available, from which 517 were successfully incorporated into a tissue microarray (TMA), as previously described [22]. Briefly, representative 0.6mm diameter cores were implanted in the TMA block using Beecher's manual tissue microarray (MP06 Beecher Instruments Inc., Sun Prairie, WI, USA).

Clinical information was available from diagnosis to death or last follow-up. Patient clinicopathological characteristics are summarized in Table 1. Median follow-up was 6.3 years (95% CI, 6.1–7.1 years).

The TMA was used to test expression of a panel of biomarkers by IHC, using the StreptAvidin Biotin Complex and EnVision methods, which have been extensively described [4]. Biomarkers tested in Nottingham included ER, PR, HER2, Ki67, Bcl2, BRCA1, BRCA2, E-Cadherin, EGFR, LKB1, MDM2, MDM4, MUC1, p53, VEGF and cytokeratin markers CK5, CK5/6, CK14, CK17 and CK18.

Staining of the biomarker panel was assessed as previously reported [4]. Cytoplasmic staining for cyclin E was centrally assessed at MD Anderson Cancer Center as previously described, using Santa Cruz clone C-19 polyclonal antibody to cyclin E [15]. TMA results were interpreted by two independent pathologists blinded to clinical outcome and assigned according to percentage of cells stained and intensity of staining. Cut-off values for all biomarkers were as reported in our previous studies [4,14].

Conventional pathological parameters as part of standard reporting for surgical specimens at Nottingham were measured, including size, grade and axillary stage (regional lymph node involvement). The policy of axillary surgery evolved according to clinical evidence-based guidelines over the 37-year period of sample collection, as previously described [21].

The primary endpoint was breast cancer-specific survival (BCSS), defined as time from diagnosis to last follow-up or death from breast cancer (i.e., excluding death due to competing causes). Secondary endpoints included disease-free survival (DFS), defined as time from diagnosis to first recurrence, and overall survival (OS), defined as time from diagnosis to last follow-up or death from any cause.

The bioinformatics software X-Tile was used to define thresholds for biomarker positivity, as previously described [23]. Biomarker expression was compared between groups by  $\chi^2$  test. Kaplan–Meier survival analysis was performed using logrank and generalized Wilcoxon tests. Multivariate analysis of BCSS was performed using the Cox regression model. Statistical significance was defined as  $p < 0.05$ . Reporting adhered to reporting recommendations for tumor marker prognostic studies (REMARK) criteria [24].

This research was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of the University of Nottingham, Nottingham University Hospitals NHS Trust and the University of Texas MD Anderson Cancer Center (MDA ref.33).

#### 5. Conclusions

This study highlights the clinical value of tumor c-cyclin E status as a strong predictor of the biological course of primary breast cancer in older patients. Those who present with c-cyclin E-negative tumors are unlikely to die of their breast cancer. Alongside existing tools such as geriatric assessment and biomarkers of treatment response such as ER positivity, c-cyclin E status may assist initial therapy decision-making in terms of intensity, duration and modality. These hypotheses warrant prospective clinical evaluation in the specific clinical context of primary breast cancer in older women.

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Article

# HDAC5 Inhibitors as a Potential Treatment in Breast Cancer Affecting Very Young Women

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**Abstract:** Background: Breast cancer in very young women (BCVY) defined as <35 years old, presents with different molecular biology than in older patients. High *HDAC5* expression has been associated with poor prognosis in breast cancer (BC) tissue. We aimed to analyze *HDAC5* expression in BCVY and older patients and their correlation with clinical features, also studying the potential of *HDAC5* inhibition in BC cell lines. Methods: *HDAC5* expression in 60 BCVY and 47 older cases were analyzed by qRT-PCR and correlated with clinical data. The effect of the *HDAC5* inhibitor, LMK-235, was analyzed in BC cell lines from older and young patients. We performed time and dose dependence viability, migration, proliferation, and apoptosis assays. Results: Our results correlate higher *HDAC5* expression with worse prognosis in BCVY. However, we observed no differences between *HDAC5* expression and pathological features. Our results showed greatly reduced progression in BCVY cell lines and also in all triple negative subtypes when cell lines were treated with LMK-235. Conclusions: In BCVY, we found higher expression of *HDAC5*. Overexpression of *HDAC5* in BCVY correlates with lower survival rates. LMK-235 could be a potential treatment in BCVY.

**Keywords:** breast cancer; young women; histone deacetylase; *HDAC5* inhibitors; LMK-235

## 1. Introduction

Breast cancer is the most common cancer among women worldwide [1]. As a percentage of all cancers in young women, breast cancer rates increase during the third and fourth decades of life, from 2% at age 20 to more than 40% by age 40. This abrupt increase is attributable to routine screening mammography. Breast cancer incidence is similar among young women in both developed and developing countries [2], but in recent years, there has been an increase in breast cancer diagnoses in very young women [3]. The distribution of breast cancer subtypes and grades changes with age, with more aggressive phenotypes among young women compared with older counterparts [4]. Basal-like and Her2-enriched breast tumors are also more common in young than in older women [5]. Scientific evidence suggests that age at breast cancer diagnosis represents an independent prognostic

survival factor [6]. Indeed, multiple studies conclude that early age at diagnosis of breast cancer is highly associated with an increased risk of recurrence and death [7].

Epigenetic modifications are reported to play an important role in the onset and progression of many diseases. Carcinogenesis can be explained by genetic alterations, but epigenetic processes such as DNA methylation, histone modifications, and non-coding RNA deregulation are also involved [8].

Histone deacetylases are essential for global acetylation patterns in the nucleus and the epigenetic regulation of gene expression [9]. Histone deacetylases (HDACs) have been stratified into classes I, IIA, IIB, III, and IV and increased expression of these isoenzymes has been observed in different tumors, also often associated with poor outcome [10–13]. It has been reported several times that their levels vary greatly in cancer cells and differ according to tumor type. Of the 18 human HDACs, HDAC5 (a class IIA HDAC) is involved in synoviocyte activation, neural regeneration and repair, differentiation of myoblasts, and recently, elevated expression of *HDAC5* has been correlated with worse prognosis in patients with breast cancer (BC) [14].

Histone deacetylase inhibitors (HDACi) are currently acquiring importance in cancer treatment, being the only approved epigenetic therapy in clinics altering histone proteins to date. HDACi have been classified into four groups depending on their structure: Hydroxamic acids, cyclic peptides, benzamides, and short chain fatty acids. The hydroxamic acids (vorinostat, belinostat, and panobinostat) have been approved by the FDA as anticancer treatments. However, they are limited by their nonspecificity, affecting all HDACs [15]. These are known as pan-HDAC inhibitors and may also cause numerous side effects due to their broader specificity. Therefore, in some cases, more selective inhibitors may be more effective in therapy [16–19].

A previous study based on evaluation of new HDAC inhibitors identified LMK235 (N-((6-(hydroxyamino)-6-oxohexyl)oxy)-3,5-dimethylbenzamide) as the most cytotoxic compound, displaying equipotent HDAC inhibition in pan-HDAC assay when compared to vorinostat. In contrast to vorinostat, LMK235 showed a novel HDAC isoform selectivity profile with a preference for HDAC4 and HDAC5 [20].

Previous group studies showed significant differences in miRNA expression [21] and methylation [22] profiles among breast cancer affecting very young women (<35 years) (BCVY) and breast cancer in older patients (>45 years) (BCO). Additionally, our group observed considerable hypomethylation of CpG regions that were regulating *HDAC5* expression in BCVY, and this methylation was related to significant *HDAC5* overexpression in BCVY patients [23]. In the present study, we analyse *HDAC5* expression in a large cohort of BC patients, to clarify the correlation between *HDAC5* overexpression and relapse and survival in BCVY and BCO and the inhibitory effect of LMK-235 in breast cancer cell lines from very young and older women with BC.

## 2. Results

### 2.1. Clinical-Pathologic Characteristics of Patients

A total of 107 patients were included, 60 were young women under 35 years (BCVY) and 47 samples from women over 45 years (BCO). The median age at breast cancer diagnosis in the young patient's group was 32 years (range, 20–35), and in the old patient's group was 69 years (range, 53–94). Hereditary cases with BRCA1 and BRCA2 mutations were excluded from the study. In BCVY cohort, the immunohistochemical analyses showed 39.9% (n = 24) of luminal patients, 11.6% (n = 7) HER2-positive, 21.6% (n = 13) luminal/HER2 and 23.3% (n = 14) triple negative. In BCO group, 59.5% (n = 28) of patients were luminal, 10.6% (n = 5) HER2-positive, 10.6% (n = 5) luminal/HER2 and 17% (n = 8) presented triple negative subtype. Median follow-up was 93.4 months (Table 1).

**Table 1.** Clinicopathological information of breast cancer (BC) samples and statistical results from the study of HDAC5 expression vs. clinicopathological features by age groups.

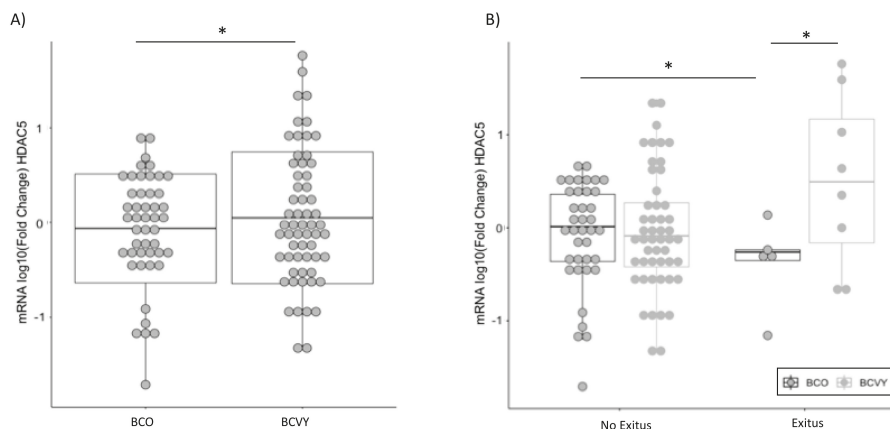
	BCVY	<i>p</i> -Value (HDAC5 ~ CP)	BCO	<i>p</i> -Value (HDAC5 ~ CP)
N	60		47	
Age mean (years ± SD)	32.1 ± 3.3		69.8 ± 9.3	
Histological subtypes (%)		0.46		0.40
Luminal A	16.6		27.6	
Luminal B	23.3		31.9	
TNBC	23.3		17.0	
Luminal/HER2	21.6		10.6	
HER2	11.6		10.6	
Unknown	3.6		2.3	
ER status (%)		0.43		0.92
ER+	60.0		74.4	
ER-	33.3		21.3	
Unknown	6.7		4.3	
PR status (%)		0.44		0.44
PR+	50.0		57.4	
PR-	43.3		38.3	
Unknown	6.7		4.3	
HER2 (%)		0.97		0.93
HER2+	31.6		21.3	
HER2-	61.6		74.5	
Unknown	6.8		4.2	
KI67 (%)		0.56		0.07
<15%	16.6		27.7	
15–30%	28.3		32.0	
>30%	38.3		27.7	
Unknown	16.8		12.6	
Grade (%)		0.16		$2.8 \times 10^{-3}$ **
I	13.2		23.3	
II	49.1		41.9	**
III	37.7		34.9	*
Unknown				
Tumour Size (%)		0.16		0.02 *
<2 cm	35.0		59.6	
2–5 cm	40.0		23.4	
>5 cm	20.0		8.5	
Unknown	5.0		8.5	
Axillary Affection (%)		0.98		0.17
POS	38.3		29.8	
NEG	56.6		63.8	
Unknown	5.1		6.4	
Exitus (%)	13.8	0.12	13.3	0.19
Relapse (%)	25.0	0.27	21.0	0.15

N: Sample size; SD: Standard deviation; TNBC: Triple negative subtype; ER: Estrogen receptor; PR: Progesterone receptor; BCVY: Breast cancer in very young women; BCO: Breast cancer in older women. P-values indicate statistics for the differences among HDAC5 expression and the different clinicopathological (CP) features included in the table by age groups. \* *p*-value < 0.05, \*\* *p*-value < 0.01.

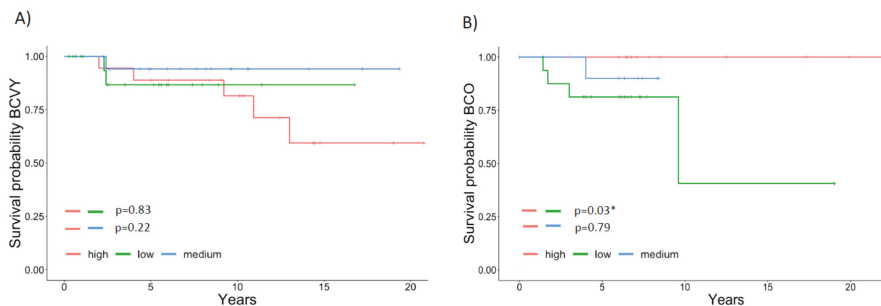
## 2.2. HDAC5 Overexpression in BCVY Patients Correlates with Poorer Prognosis

HDAC5 was significantly overexpressed in BCVY patients (*p*-value = 0.04) (Figure 1A). We found no appreciable differences among clinical features and HDAC5 expression, apart from tumor grade in BCO patients (*p*-value =  $2.8 \times 10^{-3}$ ) where the lower grades presented higher HDAC5 expression (Figure S1A). Regarding molecular subtypes, we observed higher HDAC5 expression in Luminal B and HER2 tumors from BCVY in comparison with BCO patients from the same subgroups. While BCO patients presented higher expression for Luminal A and Luminal/HER2 comparing to BCVY (Figure S1B). Despite no significant association, these results agree with higher expression of HDAC5 for poor prognostic subtypes for each patient age groups in our cohort, that were HER2 for BCVY and Luminal/HER2 for BCO patients.

We observed similar percentages of cancer death between young and old patients, 13.8% (n = 9) and 13.3% (n = 6), respectively. However, *HDAC5* was significantly overexpressed in BCVY patients that died in comparison with BCO ( $p$ -value = 0.04) and a significant *HDAC5* overexpression was observed for BCO patients that survive when compare with BCO patients that died ( $p$ -value = 0.01) (Figure 1B). These results were in line with survival studies, where Kaplan–Meier curves showed a reduced survival trend for BCVY when *HDAC5* was overexpressed (Figure 2A) whereas BCO women presented poorer survival when *HDAC5* was repressed (Figure 2B). In terms of relapse, results were not as evident as survival (results not shown). For survival and relapse studies, samples were classified in high, medium or low, according to *HDAC5* expression. Despite no significant results observed for BCVY patients, survival studies indicated an important trend of worst survival for BCVY, contrary to the results observed for BCO. In this regard, *HDAC5* overexpression in BCVY could be related with the poorer outcome at this age group. It is worth to mention that the number of BC patients that experience relapse and/or death was reduced and further studies should be addressed in order to validate this tendency observed.



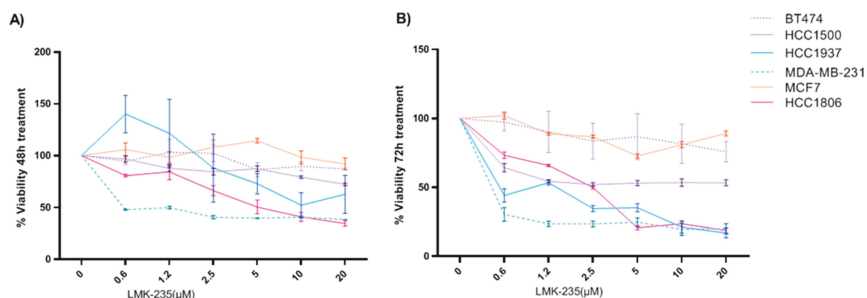
**Figure 1.** *HDAC5* expression in BCVY (n = 60) and BCO (n = 47) patients analyzed by qRT-PCR (A). *HDAC5* expression in BCVY and BCO patients regarding their status (exitus or no exitus) (B). BCVY: Breast cancer in very young women; BCO: Breast cancer in old women. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ .



**Figure 2.** Association of *HDAC5* expression and survival in breast cancer patients. Survival curves for *HDAC5* expression in BCVY (n = 42) (A) and BCO patients (n = 28) (B). *HDAC5* expression was classified in high (red), medium (blue) or low (green). BCVY: Breast cancer in very young women; BCO: Breast cancer in old women. \*  $p \leq 0.05$ .

### 2.3. LMK-235 Inhibitor Reduces Proliferation in BC Cell Lines

Breast cancer cell lines were exposed to increasing concentrations of HDAC5 inhibitor LMK-235 (0, 0.625, 1.25, 2.5, 5, 10, and 20  $\mu\text{M}$ ) for 24, 48, and 72 h. We observed that relative proliferation decreased in a dose- and time-dependent manner for some breast cancer cell lines (Figure 3). After 48 h treatment with low doses of LMK-235 (Figure 3A), cell viability of MDA-MB-231 and HCC1806 cell lines was severely compromised. Additionally, viability was notably diminished in the HCC1937 cell line after 72 h of treatment, showing similar results to MDA-MB-231 and HCC1806 cell lines (Figure 3B). Although no significant results were obtained for HCC1500 cell line after 48 h of treatment, the young cell line showed a 50% reduction in viability after 72 h of low dose LMK-235. Breast cancer cell lines that experienced significant viability reduction were triple negative subtype with the exception of HCC1500 luminal cell line established from young BC patients and showed higher reduction in comparison with the older luminal cell lines, MCF7 and BT474. Inhibitor studies reveal important response for triple negative BC cell lines to LMK-235 and for luminal cell lines from young women with BC not observed in older luminal cell lines.

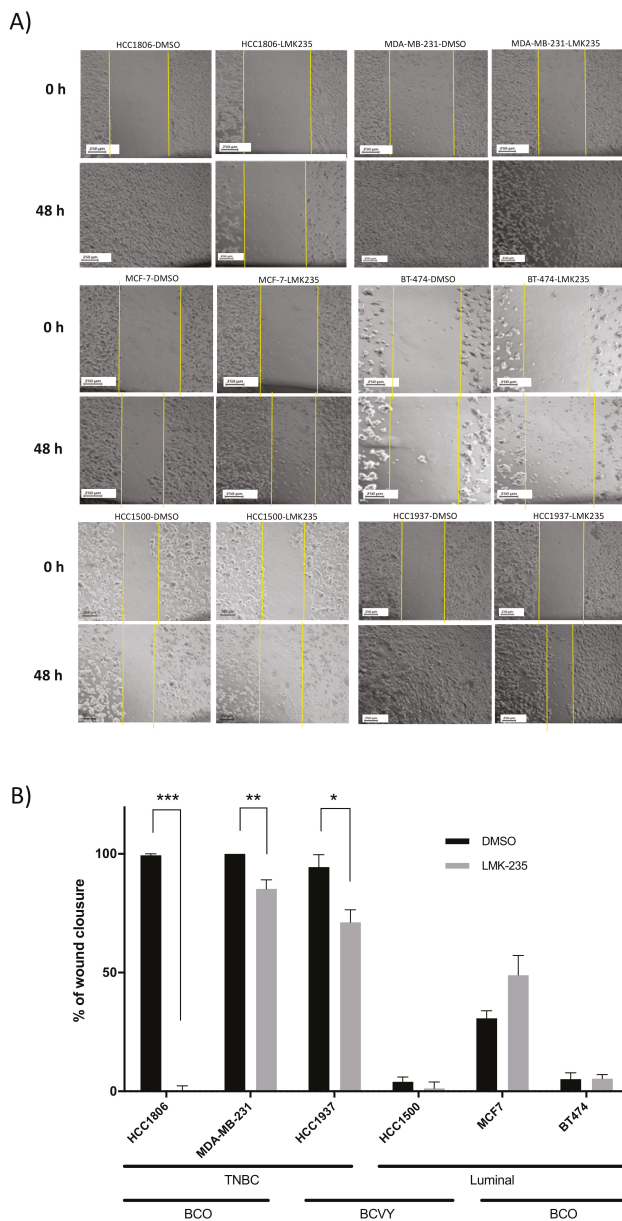


**Figure 3.** Effects of LMK-235 treatments in BC cell proliferation. Cell proliferation was determined by MTT assay for HCC1500 and HCC1937 (<35 years old) and MDA-MB-231, MCF-7, HCC1806, and BT-474 (>45 years old) cell lines that were treated with LMK-235 (0 to 20  $\mu\text{M}$ ) for 48 hours (A), 72 h (B). Points indicate the mean of at least three independent experiments and the standard deviation.

### 2.4. Reduced Migration of Breast Cancer Cell Lines Treated with LMK-235 Measured by Wound-Healing Assay

Wound-healing assays demonstrated that LMK-235 significantly inhibited migration in HCC1937 ( $p$ -value = 0.01), HCC1806 ( $p$ -value =  $4.9 \times 10^{-7}$ ) and MDA-MB-231 ( $p$ -value =  $1.2 \times 10^{-3}$ ) breast cancer cell lines after 48 h of treatment. Specifically, we observed ~23% less cell migration in the HCC1937 cell line, ~14.7% for MDA-MB-231, and also HCC1806 cell line showed a substantial reduction. HCC1500 cells treated with LMK-235 presented cell migration reduced by ~5.3%. However, the last cell line exhibited considerable cell death at 48 h of cell treatment making wound-healing analysis difficult. In contrast, breast cancer cell lines MCF-7 and BT474, both from luminal old BC patients, showed insignificant cell migration reduction when cells were treated with LMK-235 (Figure 4).

These results revealed higher cell migration and proliferation reduction in cell lines from triple negative subtypes (MDA-MB-231, HCC1806, and HCC1937), independently of age. However, we observed markedly diminished viability in the young and luminal HCC1500 cell line treated with HDAC5 inhibitor, which could not be detected in the luminal subtypes from older cell lines (MCF7 and BT474).



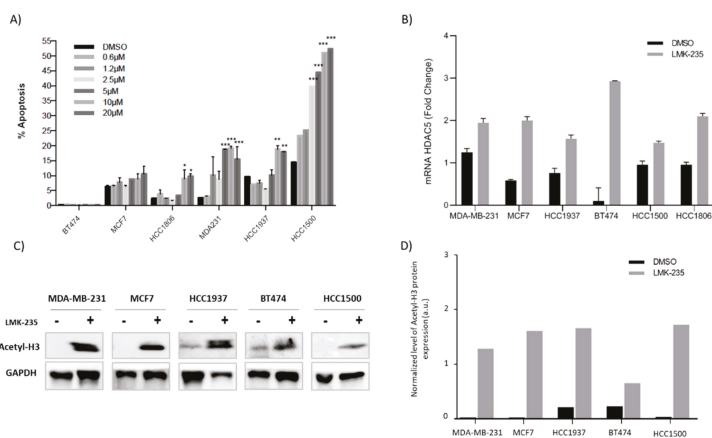
**Figure 4.** Effects of LMK-235 treatments in BC cell migration. Cell migration was assessed by scratch wound-healing assay after treatment with LMK-235 (20 μM) for 48 h. Images of cell migration at 0 h and 48 h after LMK-235 (20 μM) treatment (A); Percentage of wound closure after 48 h of LMK-235 (20 μM) treatment or DMSO/control. Three separate experiments were conducted, and representative results are shown (B). Columns mean ± SD of the percentage of wound closure in the three independent experiments. Black bars represent control DMSO cells and grey bars represent cells treated with LMK-235. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$  statistically significant. TNBC: Triple negative breast cancer; BCVY: breast cancer in very young women; BCO: Breast cancer in old women.

## 2.5. Apoptosis After LMK-235 Treatment

To confirm the results obtained by MTT, we performed an apoptosis assay by flow cytometry. After 48 h of treatment, similar results to the MTT assay were observed. Results show increasingly early apoptosis in most BC cell lines after 48 h of LMK-235 (10 and/or 20  $\mu$ M) treatment. Interestingly, and in line with previous proliferation and migration results, the most important increase in apoptosis was observed in triple negative cell lines from both BCVY and BCO. Intriguingly, LMK-235 induced a more profound increase in HCC1500 cell death for higher concentrations than in MTT assay (Figure 5A). These results agree with the wound-healing assay observations, where after 48 h of treatment most of the HCC1500 cells were dead and no wound-healing analysis could be performed.

## 2.6. Accumulation of Acetyl-H3 After LMK-235 Treatment in Breast Cancer Cell Lines

We observed increased *HDAC5* mRNA expression in all breast cancer cell lines that were treated with LMK-235 (Figure 5B). These results confirm that the HDACi does not act at the mRNA level, but rather at the protein level. LMK-235 induces an increase in mRNA expression as a positive feedback mechanism in order to increase and restore protein expression. Additionally, *HDAC5* expression was higher in control/DMSO conditions for all BC cell lines independently of their response to the HDAC5 inhibitor.



**Figure 5.** Percentages of apoptosis are shown after treatment with LMK-235 (0.6 to 20  $\mu$ M) or DMSO for 48 h. Apoptosis was analyzed by triplicated and SD was calculated (A); Expression of *HDAC5* in breast cancer cell lines treated with LMK-235 (20  $\mu$ M) and DMSO control (B); Accumulation of acetyl-histone H3 after 48 h of LMK-235 (20  $\mu$ M) treatment in breast cancer cell lines examined by western-blot. The levels of acetyl-histone H3 were determined by western blot. GAPDH was used as a loading control (C). Densitometry western blot analyzed by ImageJ software (D). \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

To investigate whether the changes in *HDAC5* mRNA expression are reflected in persistent changes at their protein levels, these were determined in breast cancer cell lines by western-blotting following treatment with LMK-235 (20  $\mu$ M) for 48 h. The levels of HDAC5 protein were barely detectable for all cell lines, as previously observed in other studies [14,24] that showed the low quantity of HDAC5 protein by western blot (data not shown). Next, we measured HDAC5 activity at the acetylation of lysine residues on histone H3 to determine the inhibitory effect of LMK-235 on HDAC5. Western-blot studies showed accumulation of acetyl-histone H3 in breast cancer cell lines after 48 h of LMK-235 treatment (Figure 5C,D). We observed acetyl-histone H3 accumulation both in luminal breast cancer cell lines (BT474, HCC1500 and MCF7) and in triple negative breast cancer cell lines (HCC1937 and MDA-MB-231). These results demonstrate that LMK-235 specifically inhibits HDACs, which catalyze



the removal of acetyl groups from N-acetyl lysine histone residues. Therefore, their inhibition produces an accumulation of acetyl-histones and acetyl-H3 is one of the most extensively modified.

### 3. Discussion

BCVY can be understood as a unique and different entity from breast cancer in older people, not only because of patients' characteristics (where aspects such as fertility preservation must take into account) but also in its biological and molecular tumor characteristics. Our group has focused for years on the study of these differential molecular characteristics in breast cancer tumors of young and old women. Previous results [23] showed us that *HDAC5* was notably overexpressed in BCVY. We validate this issue analyzing *HDAC5* overexpression in a larger cohort of young patients under 35 years and over 45, as well as studying the correlation of *HDAC5* expression levels with different clinic-pathological features. Additionally, we analyzed the effects of an HDAC5 inhibitor, LMK-235, on apoptosis, proliferation, and migration in breast cancer cell lines.

Histones are acetylated on lysine side chains, which neutralizes lysine's positive charge, leading to open chromatin structure by reducing interaction between histone and negatively charged DNA [25,26]. Thus, histone acetylation increases the accessibility of proteins such as transcription factors to promoters and enhancers, thereby mediating active gene expression. Acetylated histones also function as binding sites for numerous proteins with bromodomains, which often activate gene transcription [25]. In contrast, histone deacetylation is associated with chromatin condensation and transcriptional repression [25]. Analogous to histone methylation, histone acetylation is reversibly controlled by two large enzyme families: histone lysine acetyltransferases (KATs) and HDACs.

Turning to *HDAC5*, high expression has been correlated with poorer prognosis in patients with BC or pancreatic neuroendocrine tumors and has been attributed to oncogenic effects [27,28]. Specifically, *HDAC5* overexpression has been correlated with triple negative BC tumors [14,29]. However, our study showed higher *HDAC5* expression in BCVY than BCO samples. Next, we wonder whether *HDAC5* overexpression was correlated with relapse and survival, and other clinical and pathological data. BCVY patients with *HDAC5* overexpression showed a higher risk of exitus, whereas results were contrary for BCO that showed a significant risk of death when *HDAC5* was repressed. In agreement with these results, higher *HDAC5* expression was observed in BCVY samples that died whereas lower expression was found in BCO patients that die. Our results reinforce the hypothesis that *HDAC5* overexpression in BCVY patients is related with a poor outcome observed in this group of age. BCVY is not a usual diagnosis, so there is a limitation in the number of BCVY samples and the present study is actually the first report that includes one of the highest cohort of BCVY patients in comparison with other larger datasets (ex., TCGA and METABRIC [30,31]).

Next, we evaluated *HDAC5* expression and its correlation with different clinical and pathological features (histologic subtypes, Ki67, tumor size, grade, and axillary affection). We found no correlation between *HDAC5* expression and clinical features in BCVY. The only meaningful association observed was between *HDAC5* downregulation and higher tumor grades (II and III) in BCO. Higher tumor grades are associated with poorer prognosis and in our study with *HDAC5* repression for BCO, which agrees with survival tendencies observed at this age group where *HDAC5* repression correlates with poorer survival in BCO.

HDAC inhibitors are the most widely investigated epigenetic drugs in clinical studies [32]. In vitro studies suggest that the HDAC5 inhibitor LMK-235 could be a novel therapeutic strategy for BC treatment [14]. We evaluated the inhibitory effect of LMK-235 in breast cancer cell lines from young women and compared it with breast cancer cell lines from older counterparts. Our results demonstrated that LMK-235 induced important reduction in progression, migration, and apoptosis, not only in triple negative young and old but also in luminal young cell lines. Regarding that, we observed important viability reduction under treatment conditions for the cell lines that presented significant differences in the migration assay between control and treatment conditions. These results were observed in triple negative cell lines (from young and old patients). Thus, the reduction in cell viability correlates with a

reduction in cell migration under LMK-235 treatment. Interestingly, the luminal cell lines from old patients (BT474 and MCF7) were not affected by LMK-235 inhibitor. However, the luminal young cell line HCC1500 presented intermedium results between triple negative and luminal old cell lines. These results point out a potential breast cancer treatment not only for triple negative breast cancer but also for young patients from different molecular subtypes.

Additionally, LMK-235 response was lower in ER-positive cell lines, except for HCC1500 which presented an intermedium response in comparison with the rest of ER-positive cells, as we previously mentioned. Previous studies found LMK-235 response in triple negative BC cell lines [33–36]. However, our results showed an important response to LMK-235 in the breast cancer cell line from young women that present luminal subtype, as HCC1500. These results suggest an effect of ER in the LMK-235 treatment but must exist other mechanisms in young cell lines that increase the effect of LMK-235, which are not present in older cell lines. There was a pronounced time- and dose-dependent decrease in cell viability and migration and hence an increased apoptosis in all breast cancer cell lines from triple negative subtypes (BCVY and BCO). Furthermore, HCC1500 luminal BCVY cell line presented similar results, showing significant apoptosis rates and reduced cell viability, avoiding the possibility of analyzed migration wound-healing assays. It is worth to mention that LMK-235 treatment induces higher apoptosis in HCC1500 cell line at lower doses in comparison with the rest of cell lines. These results suggest the existence of off-target effects that increase the apoptosis at this cell line, so further studies are required to analyze the underlying mechanisms of LMK-235 inhibitor.

These results reinforce the hypothesis that *HDAC5* is involved in tumorigenesis and cancer progression reducing survival, specifically in BCVY. We propose studying HDAC5 inhibitors like LMK-235 in a larger cohort of breast cancer cell lines from young and old women and from different molecular subtypes in order to evaluate their inhibitory effect. Additionally, cell lines studies show a significant response to HDAC5i of triple negative BCO in addition to all BCVY cell lines independently of their molecular subtype.

## 4. Materials and Methods

### 4.1. Patient Selection

All samples included in the study were archived formalin-fixed paraffin-embedded (FFPE) BC tissues stored at the Hospital Clínico Universitario of Valencia, Spain. We selected 60 breast cancer samples from women under 35 years old (BCVY) and 47 samples from women over 45 (BCO). The clinical characteristics of patients are included in Table 1. This study was approved by the Institutional Health INCLIVA-Hospital Clínico Ethics Committee and informed consent was obtained from all subjects. The code of the ethical committee of the Hospital Clínico Universitario of Valencia (Spain) is 2013/128.

All patients' clinical data and tumor pathological characteristics were collected at their first visit and information about relapse and survival on subsequent visits. Tumor size and lymph node involvement were assessed using the eighth edition of the American Joint Committee on Cancer staging manual [37]. Patient clinicopathological data used in this study are shown in Table 1.

### 4.2. Breast Cancer Cell Lines Culture and Treatment

Breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cell lines were cultured in RPMI 1640 or DMEM medium supplemented with 1% L-glutamine and 10% fetal bovine serum (GIBCO, New York, NY, USA). The culture conditions were identical in all cell lines: 37 °C and 5% CO<sub>2</sub> (Table 2). Cells were seeded for 24 h before treatment with LMK-235 (Selleck Chemicals, Houston, TX, USA) or DMSO (control) for MTT assay, wound-healing, and apoptosis assay.

**Table 2.** Cell lines characteristics and culture conditions.

Cell Line	Subtype	Receptor Expression	Tumor Type	Age	Culture Medium	Conditions	Supplements
HCC 1500	Luminal	ER, PR	IDC	32	RPMI	5% CO <sub>2</sub> 37 °C	1% L-glu 10% FBS
HCC1937	Basal	EGP2	IDC	24	RPMI	5% CO <sub>2</sub> 37 °C	1% L-glu 10% FBS
MDA-MB-231	Basal	EGFR, TGF-β	Carcinoma	51	RPMI	5% CO <sub>2</sub> 37 °C	1% L-glu 10% FBS
MCF7	Luminal	ER, IGF1R	IDC	69	RPMI	5% CO <sub>2</sub> 37 °C	1% L-glu 10% FBS
BT474	Luminal	ER, PR, HER2	IDC	60	DMEM	5% CO <sub>2</sub> 37 °C	1% L-glu 10% FBS
HCC1806	Basal	EGP2	Carcinoma	60	RPMI	5% CO <sub>2</sub> 37 °C	1% L-glu 10% FBS

EGP2: Epithelial glycoprotein 2; EGFR: Epidermal growth factor receptor; TGF-β/α: transforming growth factor β/α; ER: Estrogen receptor; PR: Progesterone receptor; HER2: Hormonal estrogen receptor 2; IGF1R: Insulin growth factor binding protein; RPMI: RPMI 1640 medium; FBS: Fetal bovine serum; L-glu: L-glutamine; IDC: Invasive ductal carcinoma.

#### 4.3. Cell Proliferation Assay

The protocol used is based on a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. We seeded and cultured 2000 cells in 96-well plates. Next, cells were treated with a specific drug dose, and MTT assay was performed 24, 48, and 72 h after treatment. The absorbance of this colored solution was quantified by measuring at 590 by spectrophotometer. Each experiment was performed in triplicate and repeated at least twice. Average values for triplicates were calculated. Absorbance observed at different drug concentrations was compared with the respective non-treated controls and cell viability was calculated.

#### 4.4. Scratch Wound-Healing Assay

Cells were seeded in six-well plates at a density of  $5 \times 10^5$  cells/well and incubated overnight until they reached 70% confluence. A pipette tip was used to generate a wound in the cell layer. Cells were then treated with LMK-235 (20 μM). Each experiment was performed in triplicate and repeated at least twice. Images were obtained at 0, 24 and 48 h at the same position and percentage of cell migration was evaluated using ImageJ (LOCI, University of Wisconsin).

#### 4.5. Apoptosis Assay by Flow Cytometry

We plated 100,000 cells in 12-well plates. After 48 h of treatment, trypsinized and floating cells collected from the supernatant were centrifuged and incubated with Annexin V-FITC (BioLegend, San Diego, California, United States) and DAPI for 15 min. Apoptosis was detected using Flow Cytometry BD LSRFortessa™ (BD, Franklin Lake, NJ, USA).

#### 4.6. Western Blot

We then prepared the nuclear protein fraction of breast cancer cell lines using a nuclear extraction kit (Active Motif, Belgium, Germany). A total of 40 μg of protein were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 5% BSA and hybridized with antibodies against acetyl-H3 (1:1000) and GAPDH (1:1000) as a loading control. Immunoreactive bands were visualized by chemiluminescence (GE Healthcare, Life Science, Oslo, Norway) and captured by Image Quant™ LAS4000 (GE Healthcare, Life Science, Oslo, Norway). The band densities, normalized to the GAPDH, were analyzed with ImageJ software.

#### 4.7. RNA Extraction

Total RNA from BC cell lines was isolated using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland), following the manufacturer's protocol. Total RNA from FFPE samples was isolated using RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems™ by Life Technologies™, Carlsbad, CA, USA), following the manufacturer's protocol. RNA concentration was measured using a NanoDrop ND 2000-UV-vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

#### 4.8. Gene Expression by qRT-PCR

Gene expression of *HDAC5* and *GAPDH* as endogenous control was carried out by quantitative real time-PCR (qRT-PCR) in RNA extracted from FFPE samples. We used the TaqMan@Gene Expression Assays (Applied Biosystems™ by Life Technologies™, Carlsbad, CA, USA). Normalization was done with *GAPDH*. The data were managed using the QuantStudio Design & Analysis Software v1.4 (Applied Biosystems™ by Life Technologies™, Carlsbad, CA, USA). Relative expression was calculated using the comparative Ct method and obtaining the fold-change value ( $\Delta\Delta Ct$ ).

#### 4.9. Statistical Analysis

All statistical analysis was performed using R Bioconductor (<https://www.bioconductor.org/>). To determine differences between *HDAC5* expression and breast cancer patients/cells from different ages we performed a Wilcoxon Rank Sum test. Results were considered significant when  $p$ -value < 0.05. OS and RFS were performed using follow-up data from BC patients to analyze the prognostic value of *HDAC5* expression in terms of relapse and survival. Patients were divided into three groups according to the distribution of the log<sub>10</sub> *HDAC5* Fold change: high expression (> 0.2 log<sub>10</sub> Fold change), medium (> -0.2 and <0.2) and low (< -0.2). RFS and OS studies were performed using *survival* R package (<https://CRAN.R-project.org/package=survival>).

### 5. Conclusions

Taken together, the results provide insights into the biology of breast cancer in younger women and support our hypothesis that breast tumors of younger women activate molecular pathways related to increased aggressiveness, such as *HDAC5*. Our findings are consistent with those previously published by Li et al. [14], confirming that *HDAC5* promotes proliferation, invasion, and migration in human breast cancer. We also demonstrate that *HDAC5* overexpression in BCVY correlates with lower survival rates.

In our study, we show that targeted treatment with *HDAC5* inhibitor LMK-235 reduces migration and proliferation of tumor cells and increases apoptosis in triple negative breast cancer cell lines, as previous results demonstrated, and as a novelty in luminal cell lines from younger BC patients. In summary, our findings show, for the first time, a potential treatment specific for breast cancer affecting very young patients. However, more efforts should be addressed to analyze its therapeutic role.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/12/2/412/s1>, Figure S1: *HDAC5* expression according to tumor grade in breast cancer affecting very young patients (BCVY) and old patients (BCO) (A). *HDAC5* expression according to breast cancer age groups and molecular subtypes (B). \* $P \leq 0.05$ , \*\* $P \leq 0.01$ .

**Author Contributions:** G.R., M.T.M. and A.L. conceived the study. G.R., M.T.M., P.E. and M.P.-C. supervised the study. M.T.M. and S.S.O. coordinate and designed all the aspects of the work. S.S.O. and E.T. contributed in experimental and computational analysis. M.A., A.F. and M.N. contributed in the cell lines experiments. E.A. and O.B. contributed in the pathology aspects. A.L., B.B., J.M.C., C.H., I.C. and M.T.M. contributed in clinical patient data selection. S.S.O. and M.T.M. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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Article

# Clinical Significance of Lymph-Node Ratio in Determining Supraclavicular Lymph-Node Radiation Therapy in pN1 Breast Cancer Patients Who Received Breast-Conserving Treatment (KROG 14-18): A Multicenter Study

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**Abstract:** This study evaluated the clinical significance of the lymph-node ratio (LNR) and its usefulness as an indicator of supraclavicular lymph-node radiation therapy (SCNRT) in pN1 breast cancer patients with disease-free survival (DFS) outcomes. We retrospectively analyzed the clinical data of patients with pN1 breast cancer who underwent partial mastectomy and taxane-based sequential adjuvant chemotherapy with postoperative radiation therapy in 12 hospitals ( $n = 1121$ ). We compared their DFS according to LNR, with a cut-off value of 0.10. The median follow-up period was 66 months (range, 3–112). Treatment failed in 73 patients (6.5%) and there was no significant difference in DFS between the SCNRT group and non-SCNRT group. High LNR ( $>0.10$ ) showed significantly worse DFS in both univariate and multivariate analyses (0.010 and 0.033, respectively). In a subgroup analysis, the effect of SCNRT on DFS differed significantly among patients with LNR  $> 0.10$  ( $p = 0.013$ ). High LNR can be used as an independent prognostic factor for pN1 breast



cancer patients treated with partial mastectomy and postoperative radiotherapy. It may also be useful in deciding whether to perform SCNRT to improve DFS.

**Keywords:** breast cancer; radiotherapy; lymph-node ratio; disease-free survival

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## 1. Introduction

In addition to surgery, chemotherapy and radiotherapy are both important in curative breast cancer treatment. Radiotherapy has a significant role in the removal of microscopic tumor cells from remnant breast tissue after breast-conserving surgery [1]. Radiotherapy is used to treat not only the remaining breast tissue, but also the tumor cells in the regional lymphatic system, including the axillary and internal mammary lymph nodes. This prevents locoregional failure in patients with breast-conserving surgery and lymph-node metastasis after axillary-lymph-node dissection. It can also reduce distant metastasis [2]. The Danish Breast Cancer Cooperative Group reported the benefits of radiation therapy targeting the supraclavicular area in high-risk breast cancer patients [3,4]. The current guidelines generally recommend that elective nodal irradiation (ENI) be applied to the regional lymphatics as well as the whole breast in locally advanced breast cancer [5–7].

The use of ENI in pathological N1 breast cancer patients is still controversial. Taxane-based chemotherapy has been used as an effective adjuvant therapy for breast cancer for decades, reducing the importance of elective nodal irradiation in low-risk breast cancer [8–10]. There is no consensus on whether ENI should be administered to patients with a low risk of regional lymphatic metastasis, such as those with N1 breast cancer [6,11–13]. The current National Comprehensive Cancer Network (NCCN) guidelines also recommend that ENI be strongly considered in N1 breast cancer [14].

In a study based on existing Korean Radiation Oncology Group (KROG) 14-18 data, supraclavicular lymph-node radiation therapy (SCNRT) was ineffective in N1 breast cancer patients undergoing taxane-based chemotherapy [15]. However, several studies have reported that various risk factors affect the outcomes of patients with N1 breast cancer, and claimed that ENI can be beneficial in patients with N1 breast cancer, depending on the risk factors present [16–18]. The lymph-node ratio (LNR), defined as the proportion of positive axillary lymph nodes among the total number of axillary lymph nodes removed, is recognized as one of these risk factors. Although the absolute number of axillary lymph nodes affected by metastasis is associated with a poor prognosis in the American Joint Committee on Cancer (AJCC) guidelines, axillary-lymph-node dissection techniques may differ across different institutions [19]. Therefore, the number of lymph nodes removed may differ, even when patients have the same numbers of metastatic lymph nodes [20]. Several studies have investigated whether LNR can be used to ensure more accurate nodal staging [19–21]. In a previous single-center retrospective study that examined the relationship between LNR and SCNRT, LNR was found to have utility as an indicator of the suitability of SCNRT [22]. Against this background, it was investigated whether LNR can be used as an index of the suitability of SCNRT in multicenter studies.

## 2. Materials and Methods

### 2.1. Study Design and Patients

In this study, the records of patients diagnosed with N1 breast cancer who underwent breast-conserving surgery between January 2006 and December 2010 at one of 12 hospitals that are members of KROG were examined. Patients who underwent Adriamycin/Taxol (AT) chemotherapy and post-lumpectomy radiotherapy for N1 breast cancer within this period were included in this study. The eligibility criteria were patients with N1 breast cancer who underwent breast-conserving surgery and axillary-lymph-node dissection, who completed postoperative AT chemotherapy and radiotherapy as planned, and for whom information regarding the pathological features of the tumor was available.

The exclusion criteria were patients who received neoadjuvant chemotherapy or chemotherapy other than AT, had a previous history of malignancy, or were male. Patients with fewer than 10 dissected lymph nodes in total were also excluded from the study to ensure accurate lymph node evaluation. The Institutional Review Board of each participating hospital approved the current study.

The patient data collected were age; the pathological features of each tumor, such as the tumor size, number of tumors, and resection margin; number of positive lymph nodes; histological grade; presence of lymphovascular invasion; and expression status of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Positivity for ER or PR was defined as an Allred score of 3–8 on immunohistochemistry (IHC). HER2 positivity was defined as either 3+ on IHC staining or 2+ on IHC with a positive fluorescence in situ hybridization or chromogenic in situ hybridization signal. The molecular subtype of each breast cancer was categorized as follows: ER+ or PR+, and HER2– (luminal A); ER+ or PR+, and HER2+ (luminal B); ER–, PR–, and HER2+ (HER2 enriched); or ER–, PR–, and HER2– (triple negative). The optimal cut-off value for LNR was determined with an analysis of the area under the curve (AUC) of a receiver-operating characteristic (ROC) curve. A value of 0.10, for which the sensitivity and specificity were highest, was chosen as the optimal cut-off point for LNR.

## 2.2. Treatments

In this study, patients who had undergone more than 10 lymph-node dissections after axillary-lymph-node dissection were evaluated. The patients were treated with adjuvant chemotherapy consisting of doxorubicin and cyclophosphamide, followed by paclitaxel or docetaxel, and then by the appropriate adjuvant hormone therapy based on the presence of hormone receptors and the HER2 receptor. All patients underwent whole-breast irradiation (WBI) and tumor-bed boost, and SCNRT was selected according to each institution's policy or physician's preference.

The dose of irradiation for the whole breast was 45.0–60.4 Gy at 1.8–3.0 Gy per fraction and the dose for the tumor bed was 4.0–19.8 Gy at 1.8–4.0 Gy per fraction. The radiation dose to the supraclavicular lymph nodes (SCN) was 45.0–50.4 Gy at 1.8–2.0 Gy per fraction. The borders of each field of WBI or WBI+SCNRT were defined differently in the 12 hospitals according to each institutional policy. The axillary lymph nodes were not intentionally irradiated, but level I and a proportion of level II axillary lymph nodes were covered during WBI while a proportion of the level II and level III axillary lymph nodes and the SCN were irradiated during SCNRT. The inclusion of the internal mammary lymph node in the radiation field was determined according to each hospital's policy, considering the location of the primary tumor, pathological findings, and status of the metastatic lymph nodes.

## 2.3. Follow-Up and Endpoints

Disease-free survival (DFS) was defined as the time from the date of diagnosis to any recurrence. The patients were followed up on every 3–6 months after surgery with history and physical examinations in each hospital. Mammography was performed every 12 months. Additional imaging studies were performed in patients with suspicious clinical signs or symptoms.

## 2.4. Statistical Analysis

Data were analyzed with SPSS ver. 20.0 for Windows (SPSS Inc., Chicago, IL, USA). The Kaplan–Meier method was used to analyze disease-free survival (DFS) and statistical significance was determined with a log-rank test. Cox's stepwise regression analysis was used for the multivariable analysis. Statistically significant variables in the univariate analysis ( $p < 0.05$ ) were included in the Cox's regression model. Statistical significance was set at  $p < 0.05$ .

## 3. Results

The characteristics of the patients with pN1 breast cancer are summarized in Table 1. In total, 1121 patients satisfied the inclusion criteria for this study and were enrolled. Among them, 745 patients did

not undergo SCNRT and 376 patients did undergo SCNRT. The presence of an extensive intraductal component (EIC), lymphovascular invasion (LVI), number of positive lymph nodes, LNR, and the presence of an extracapsular extension (ECE) occurred significantly more frequently in the SCNRT group than in the non-SCNRT group. LNR was statistically independent of other prognostic factors, such as age, type of surgery, T stage, resection margin, LVI, molecular subtype, and histological grade.

**Table 1.** Patient characteristics.

Characteristics	Number of Patients (%)		p Value	
	Non-SCNRT (n = 745)	SCNRT (n = 376)		
Age (years)	≤45	442 (59.3)	221 (58.8)	0.859
	>45	303 (40.7)	155 (41.2)	
OP site	Left	343 (46.0)	193 (51.3)	0.094
	Right	402 (54.0)	183 (48.7)	
Pathology	IDC	702 (94.2)	346 (92.0)	0.157
	Others	43 (5.8)	30 (8.0)	
T stage	T1	388 (52.1)	181 (48.1)	0.282
	T2	351 (47.1)	192 (51.1)	
	T3	6 (0.8)	2 (0.5)	
	T4	0 (0.0)	1 (0.3)	
Number of tumors	Single	615 (82.6)	318 (84.6)	0.392
	Multiple	130 (17.4)	58 (15.4)	
Resection margin	Clear	682 (92.2)	351 (93.9)	0.588
	Less than 1 mm	51 (6.9)	20 (5.3)	
	Positive	7 (0.9)	3 (0.8)	
EIC	Unknown	5	2	0.025
	(−)	344 (71.2)	225 (63.9)	
	(+)	139 (28.8)	127 (36.1)	
LVI	Unknown	262	24	<0.001
	(−)	360 (49.0)	81 (24.0)	
	(+)	375 (51.0)	256 (76.0)	
HG	Unknown	10	39	0.725
	I or II	452 (61.9)	235 (63.0)	
	III	278 (38.1)	138 (37.0)	
Anti-HER2 therapy	Unknown	15	3	0.644
	(−)	678 (91.0)	339 (90.2)	
	(+)	67 (9.0)	37 (9.8)	
Dissected LNs	<20	456 (61.2)	245 (65.2)	0.197
	≥20	289 (38.8)	131 (34.8)	
Number of positive LNs	1	509 (68.5)	121 (32.2)	<0.001
	2	161 (21.7)	145 (38.6)	
	3	73 (9.8)	110 (29.3)	
	Unknown	2	0	
LNR	≤0.10	599 (80.6)	189 (50.3)	<0.001
	>0.10	144 (19.4)	187 (49.7)	
	Unknown	2	0	
ECE	(−)	369 (52.4)	92 (31.8)	<0.001
	(+)	335 (47.6)	197 (68.2)	
	Unknown	41	87	
Hormone therapy	(−)	172 (23.1)	86 (22.9)	0.917
	(+)	571 (76.9)	290 (77.1)	
	Unknown	2	0	
Molecular subtype	Luminal A	498 (67.0)	247 (65.7)	0.19
	Luminal B	71 (9.5)	49 (13.0)	
	HER2-enriched	53 (7.1)	17 (4.5)	
	Triple negative	122 (16.4)	63 (16.8)	
	Unknown	1	0	

Abbreviations: SCNRT, supraclavicular lymph node radiation therapy; OP, operation; IDC, invasive ductal carcinoma; EIC, extensive intraductal component; LVI, lymphovascular invasion; HG, histologic grade; HER2, human epidermal growth factor receptor 2; LNR, lymph-node ratio; ECE, extracapsular extension.

The median follow-up time was 66 months (range, 3–112). The overall 5-year DFS was 93.7%. The 5-year DFS in the subgroup was 92.8% in the SCNRT group and 94.1% in the non-SCNRT group. The patterns of failure are shown in Table 2. Distant metastasis was the major pattern of failure, and regional recurrence limited to the SCN occurred in less than 1% of the total patients.

**Table 2.** Patterns of failure.

Outcome	No. Patients (%)
Follow-up (months)	
Median (range)	66 (3–112)
Patterns of failure	
NED	1048 (93.5)
LR only	8 (0.7)
RR only	5 (0.4)
DM only	45 (4.0)
LR + DM	1 (0.1)
RR + DM	11 (1.0)
LR + RR + DM	3 (0.3)

Abbreviations: NED, no evidence of disease; LR, local recurrence; RR, regional recurrence; DM, distant metastasis.

A univariate analysis of DFS showed that T stage, LVI, histological grade, luminal type, and LNR were significant factors affecting DFS. In a multivariate analysis of these factors, T stage, LVI, histological grade, and LNR significantly affected DFS (Tables 3 and 4).

A subgroup analysis according to SCNRT was performed to analyze the risk of recurrence according to differences in LNR. In this analysis, the risk of recurrence differed significantly according to LNR in the non-SCNRT group, but there was no such difference in the SCNRT group (Figure 1). The use of SCNRT reduced the difference in the incidence of recurrence according to LNR.

**Table 3.** Univariate analysis of disease-free survival.

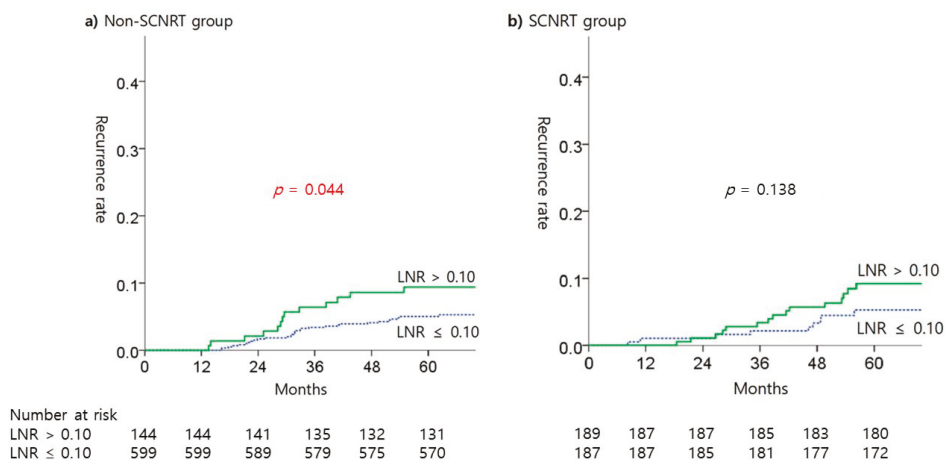
Characteristics		No. (%)	5-Year DFS	<i>p</i> Value
T stage	T1	569 (50.7)	97.1	<0.001
	T2–4	552 (49.2)	90.2	
Number of tumors	Single	933 (83.2)	93.9	0.953
	Multiple	188 (16.8)	93.1	
Resection margin	≥1 mm	1033 (92.1)	93.8	0.634
	<1 mm	81 (7.2)	92.5	
EIC	(–)	569 (50.8)	94.8	0.213
	(+)	266 (23.7)	91.8	
LVI	(–)	441 (39.3)	97.0	0.001
	(+)	631 (56.3)	91.4	
HG	I or II	687 (61.3)	96.1	<0.001
	III	416 (37.1)	89.9	
LNR	≤0.10	788 (70.3)	94.9	0.010
	>0.10	331 (29.5)	90.8	
ECE	(–)	461 (41.1)	92.7	0.110
	(+)	532 (47.5)	94.9	
Molecular Subtype	Luminal A	463 (41.3)	96.0	0.008
	Non-luminal A	657 (58.6)	82.1	

Abbreviations: EIC, extensive intraductal component; LVI, lymphovascular invasion; HG, histologic grade; LNR, lymph-node ratio; ECE, extracapsular extension.

**Table 4.** Multivariate analysis of disease-free survival.

Characteristics		Cox Regression Model		p Value
		Hazard Ratio	95% CI	
T stage	T1 vs. T2–4	2.628	1.489–4.638	0.001
LVI	(–) vs. (+)	1.92	1.071–3.441	0.028
HG	I or II vs. III	2.288	1.349–3.880	0.002
LNR	≤0.10 vs. >0.10	1.689	1.043–2.737	0.033
Molecular subtype	Luminal A vs. non-luminal A	1.029	0.695–2.121	0.496

Abbreviations: LVI, lymphovascular invasion; HG, histologic grade; LNR, lymph-node ratio.



**Figure 1.** Kaplan-Meier estimates of recurrent rate according to LNR in SCNRT treatment subgroups.

**4. Discussion**

Although many studies have reported that ENI is not required by patients with pN0 breast cancer, it is widely accepted that postoperative locoregional radiation therapy reduces locoregional recurrence and mortality in patients with lymph-node-positive breast cancer [23]. However, its effectiveness in N1 breast cancer patients treated with systemic chemotherapy is still unclear [24,25]. In this context, the NCCN guidelines recommend the use of SCNRT in patients with N1 breast cancer as for level of evidence IIB [14].

Appropriate chemotherapy for breast cancer patients not only prevents systemic metastasis of the cancer, but also reduces the risk of locoregional recurrence [26]. The recurrence rate after treatment has been steadily declining with the development of surgical and adjunct therapies, and questions about the utility of SCNRT for early breast cancer patients have begun to emerge [27]. The frequency of adverse effects, such as lymphedema, increases when SCNRT is used with systemic chemotherapy. A previous study based on the KROG 14–18 patient data used in the present study showed that SCNRT was unnecessary in the N1 patient group and increased adverse effects [15,28]. In the case of another side effect, brachial plexopathy, the risk showed 1.3% in the conventional 50 Gy SCNRT group, but the risk was increased with adjuvant chemotherapy or total dose over 50 Gy to the brachial plexus [29].

Although the risk is low, treatment failure in N1 breast cancer occurs in regional and distant areas, and may be related to microscopic tumor cells in the regional lymphatic system, which can be removed with SCNRT. SCNRT still plays an important role in breast cancer treatment within this context. This may justify the use of SCNRT in selected patients with high-risk N1 breast cancer, rather than in all patient groups. Using SCNRT for N1 breast cancer is an interesting issue in the field of radiation oncology, and several studies have recommended that SCNRT be performed after various risk factors

are considered, but not in all patient groups [17,18]. SCNRT can entail radiation side effects, such as lymphedema, brachial plexopathy, and radiation pneumonitis, so it should not be performed for small potential benefits alone. The benefits of SCNRT and its adverse effects in N1 breast cancer patients are highly controversial, and are affected by other modalities, such as the chemotherapy regimen and tumor characteristics. Therefore, its benefits should be clarified in a prospective study.

In this study, the utility of LNR was investigated as a factor to be considered in selecting SCNRT as a treatment for N1 breast cancer patients. Several studies have reported that nodal staging using LNR can predict the prognosis more effectively than conventional AJCC nodal staging [19,20,30]. In patients with N1 breast cancer with a relatively small number of metastatic lymph nodes, the prognosis can vary according to the total number of dissected lymph nodes, even in patients with the same number of metastatic lymph nodes. Therefore, this study investigated whether the utility of SCNRT can be estimated from LNR. This study was conducted with KROG 14–18 patients, and previous studies using the same patient group showed that SCNRT was ineffective in N1 breast cancer patients [15]. In the present study, it was demonstrated that SCNRT may be beneficial in patients with high LNRs, despite the use of taxane-based chemotherapy.

This study had several limitations. In addition to the limitations of all retrospective studies, there was no definitive standard according to which all institutions decided whether or not to administer SCNRT. Similarly, internal mammary node irradiation was also applied without clear criteria, which blurred the conclusions of this study. The difficulty in evaluating adverse effects was another limitation of this study. It is well known that SCNRT induces lymphedema. According to preliminary findings reported by Coen et al., the addition of regional lymph-node radiotherapy significantly increased the risk of lymphedema from 1.8% to 8.9% ( $p = 0.0001$ ) [28]. When considering the use of SCNRT, clinicians should weigh the potential benefits of SCNRT in disease control against the increased risk of lymphedema. A previous study based on existing KROG 14–18 data showed that lymphedema occurred in 16.6% of patients after WBI+ and SCNRT, but in only 10.7% of patients after WBI alone ( $p = 0.04$ ) [15]. To compare the benefits of SCNRT, a prospective study is required to establish more precise criteria. In this study, regional recurrence in the SCN, which is the main target of SCNRT, occurred in only 1% of patients. This is a very low recurrence rate upon which to base the claim that SCNRT should be performed in all patients. It is difficult to accept that SCNRT should be used to control recurrence, which occurs in only 1% of patients, given its adverse effects mentioned above. This study has shown that SCNRT also significantly reduced the risk of distant metastasis, as well as regional lymph-node recurrence. This may be because SCNRT controls the tumor burden in the SCN region, which may be the seed bed of distant metastases. Distant metastasis is the main failure pattern in N1 breast cancer patients, so this study has demonstrated that SCNRT has some therapeutic benefits over and above the control of regional recurrence. Lastly, the fact that there are characteristic differences between SCNRT group and non-SCNRT group is a weakness of the study. These characteristics are significantly higher in the SCNRT group and are generally known as poor prognosis factors, such as LVI, LNR, and ECE [13]. Despite these poor prognosis factors in the SCNRT group, there was no difference in recurrence rate between the two groups and it can be interpreted as indicating the effect of SCNRT.

Despite the several limitations described above, this multicenter study suggests that the use of SCNRT in patients receiving taxane-based chemotherapy may be beneficial if SCNRT is performed selectively. The study is important because it offers another direction of selective SCNRT for N1 breast cancer. Selective SCNRT can reduce unnecessary radiation exposure in N1 breast cancer patients and reduce the recurrence rate in appropriate patients, with a more patient-specific treatment.

## 5. Conclusions

In this DFS analysis, the patients with high LNR ( $>0.10$ ) showed benefit with DFS outcomes by SCNRT. This study has shown that LNR can be used as an independent prognostic factor in patients with N1 breast cancer and as a useful index when determining whether to perform SCNRT. However,

we did not establish whether the benefits of this treatment are sufficient to risk the associated adverse effects. Prospective studies are required to examine these issues.

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Article

# ERCC1 Is a Predictor of Anthracycline Resistance and Taxane Sensitivity in Early Stage or Locally Advanced Breast Cancers

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**Abstract:** Genomic instability could be a beneficial predictor for anthracycline or taxane chemotherapy. We interrogated 188 DNA repair genes in the METABRIC cohort ( $n = 1980$ ) to identify genes that influence overall survival (OS). We then evaluated the clinicopathological significance of ERCC1 in early stage breast cancer (BC) (mRNA expression ( $n = 4640$ ) and protein level,  $n = 1650$  (test set), and  $n = 252$  (validation)) and in locally advanced BC (LABC) (mRNA expression, test set ( $n = 2340$ ) and validation (TOP clinical trial cohort,  $n = 120$ ); and protein level ( $n = 120$ )). In the multivariate model, ERCC1 was independently associated with OS in the METABRIC cohort. In ER+ tumours, low ERCC1 transcript or protein level was associated with increased distant relapse risk (DRR). In ER–tumours, low ERCC1 transcript or protein level was linked to decreased DRR, especially in patients who received anthracycline chemotherapy. In LABC patients who received neoadjuvant anthracycline, low ERCC1 transcript was associated with higher pCR (pathological complete response) and decreased DRR. However, in patients with ER–tumours who received additional neoadjuvant taxane, high ERCC1 transcript was associated with a higher pCR and decreased DRR. High ERCC1 transcript was also linked to decreased DRR in ER+ LABC that received additional neoadjuvant taxane. ERCC1 based stratification is an attractive strategy for breast cancers.

**Keywords:** ERCC1; anthracycline resistance; taxane sensitivity

## 1. Introduction

Anthracycline and taxane based adjuvant and neoadjuvant chemotherapies are standard approaches in the management of early stage or locally advanced breast cancers to reduce distant recurrence and improve survival [1–3]. Moreover, the recent development of multi-parameter gene-expression assays, largely based on proliferation biomarkers, has facilitated the selection of patients who are most likely to benefit from systemic chemotherapy [4]. However, despite the genomic based selection, not all patients benefit from chemotherapy. In addition, chemotherapy related toxicity (such as anthracycline induced cardiotoxicity/leukaemia and taxol induced irreversible peripheral

neuropathy) can adversely impact overall outcomes. Therefore, the development of anthracycline and/or taxane specific predictive biomarkers is desirable.

Chromosomal instability (CIN) that alters chromosome number or structure is a hallmark of cancer including breast tumours [5–7]. Whilst genomic instability is a key driver of CIN, dysfunctional mitotic mechanisms, such as defective spindle assembly checkpoints and defective sister chromatid cohesions can also promote chromosomal instability [5–7]. Tumours with impaired DNA repair capacity and CIN are sensitive to DNA damaging chemotherapeutics. In early breast cancer patients, duplication of chromosome 17 centromere enumeration probe (Ch17CEP), a CIN marker, was previously shown to be a strong predictor of benefit from anthracycline adjuvant chemotherapy in a prospective clinical trial [8]. On the other hand, in a meta-analysis by the early breast cancer trialists collaborative group (EBCTCG), benefit from taxanes (paclitaxel, docetaxel) based chemotherapy was most evident in chromosomally stable low grade breast cancers [9,10]. In addition, CIN has been shown previously to predict paclitaxel resistance in ovarian cancer patients [11].

Chemotherapy induced or radiotherapy induced DNA damage is processed by various DNA repair pathways in cells. Emerging data provides strong evidence that overexpression of DNA repair factors can also contribute to therapeutic resistance in cancers [12]. DNA adducts induced by chemotherapy (such as by platinum and cyclophosphamide) are processed through the nucleotide excision repair (NER) pathway. NER is a highly conserved, versatile and robust. NER is a complex pathway requiring several proteins and their interacting partners. Although complex, two sub-pathways of NER have been described: The transcription-coupled nucleotide excision repair (TC-NER) pathway, that targets lesions specifically in the transcribed strand of expressed genes, and the global genome nucleotide excision repair (GG-NER) pathway, that deals with lesions in the rest of the genome. Although these NER sub-pathways are complex, basic steps in GG-NER include DNA damage recognition (by XPC-HR23B complex), lesion demarcation and verification by TFIIH complex (Cdk7, Cyclin H, MAT1, XPB, XPD, p34, p44, p52 and p62), assembly of a pre-incision complex (RPA, XPA and XPG), DNA opening by XPB and XPD helicases, dual incision (by ERCC1–XPF and XPG endonucleases), release of the excised oligomer and finally repair synthesis to fill in the resulting gap (RPA, RFC, PCNA, Pol  $\delta/\epsilon$ , and ligation by ligase I) [13,14]. In TC-NER, translocating RNA polymerase II detects lesions in the template. A role for ERCC8 (CSA) and ERCC6 (CSB) has also been suggested in DNA damage recognition in TC-NER. Subsequent steps in TC-NER are similar to GC-NER.

ERCC1 protein is a critical player in NER. ERCC1 is non-catalytic but associates with XPF endonuclease (also known as ERCC4) to form the ERCC1–XPF heterodimer. The ERCC1–XPF heterodimer cleaves and facilitates the removal of bulky lesions, such as those induced by platinum chemotherapy [15,16]. In addition the ERCC1–XPF heterodimer also has essential roles in other DNA repair pathways, such as DNA recombinational repair and inter-strand crosslink repair [17,18]. ERCC1 and XPF siRNA depletion was previously shown to increase cisplatin sensitivity in non-small lung [19] and breast cancer cells [20]. In a mouse xenograph model, ERCC1-deficient melanoma cells were also observed to be 10-fold more sensitive to cisplatin than ERCC1-proficient cells [21]. ERCC1 as a marker of chemotherapy resistance has been well described in other solid tumours, including lung, colorectal, head, neck, gastric, bladder and ovarian cancers [22–26]. Given the critical role of ERCC1 in genomic integrity, in the current study, we evaluated the role of ERCC1 as a biomarker in breast cancers.

## 2. Results

### 2.1. ERCC1 Transcript Is a Predictor of Tumour Grade and Chromosomal Instability in Early Stage breast cancers (BCs)

A large body of clinical evidence confirms that high grade BCs is associated with chromosomal instability. Given the critical role of ERCC1 in NER, DSB repair, ICL repair and chromosomal stability, we evaluated ERCC1 transcripts in the METABRIC Cohort. A low level of ERCC1 transcript was significantly associated with higher grade cancer, whereas low grade tumours were common in high ERCC1 tumours (Table 1) ( $p$  values < 0.0001). Low ERCC1 transcript was also associated with

Ki67 positivity, ER-, PAM50 Luminal B, Pam50 Her2, Pam50 basal and Genufu ER+/HER- (high proliferation) tumours. On the other hand, low grade, Ki67 negative, PAM50 Luminal A and Genufu ER+/HER- (low proliferation) were more common in tumours with high *ERCC1* transcript (all *p* values < 0.0001). To evaluate associations with chromosomal stability we investigated *ERCC1* in various integrative molecular cluster (intClust) phenotypes described in the METABRIC cohort. Low *ERCC1* transcript was linked to genomically unstable intClust.10 phenotype whereas high *ERCC1* was associated with chromosomally stable intClust.3, 4, 7 and 8 tumours. Together, the data provides the first clinical evidence that low *ERCC1* is a marker of chromosomal instability and aggressive phenotype in BCs.

**Table 1.** Clinicopathological significance of *ERCC1* mRNA expression in breast cancers.

	<i>ERCC1</i> mRNA Expression		<i>p</i> -Value	* <i>p</i> -Value(Adjusted)
	Low	High		
<b>(A) Pathological Parameters</b>				
Tumour Size				
≤1cm	43 (4.5%)	43 (4.4%)	0.481	5.2910
>1–2cm	247 (25.7%)	279 (28.8%)		
>2–4cm	620 (64.5%)	601 (62.0%)		
>4cm	51 (5.3%)	46 (4.7%)		
Tumour Grade				
1	35 (3.7%)	130 (14.1%)	$4.4 \times 10^{-37}$	<0.00001
2	305 (32.0%)	460 (49.8%)		
3	612 (64.3%)	334 (36.1%)		
Lymph Node Group				
Negative	486 (49.8%)	528 (54.2%)	0.051	0.0623
Positive	490 (50.2%)	446 (45.8%)		
Histological Types				
IDC-NST	837 (85.8%)	704 (72.3%)	$1.33 \times 10^{-15}$	<0.00001
Medullary Carcinoma	20 (2.0%)	12 (1.2%)		
Invasive special type	104 (10.7%)	247 (25.4%)		
Invasive others	15 (1.5%)	11 (1.1%)		
Ki67 Expression				
Negative	375 (38.4%)	600 (61.6%)	$1.37 \times 10^{-24}$	<0.00001
Positive	601 (61.6%)	374 (38.4%)		
P53 Mutation				
Wild type	325 (82.9%)	383 (92.3%)	$4.9 \times 10^{-5}$	<0.00001
Mutant	67 (17.1%)	32 (7.7%)		
ER Expression				
Negative	332 (34.0%)	126 (12.9%)	$4.8 \times 10^{-28}$	<0.00001
Positive	644 (66.0%)	848 (87.1%)		
PAM 50 Luminal A				
Negative	770 (78.9%)	466 (48.1%)	$4.45 \times 10^{-45}$	<0.00001
Positive	206 (21.1%)	502 (51.9%)		
PAM 50 Luminal B				
Negative	684 (70.1%)	775 (80.1%)	$3.68 \times 10^{-7}$	<0.00001
Positive	292 (29.9%)	193 (19.9%)		

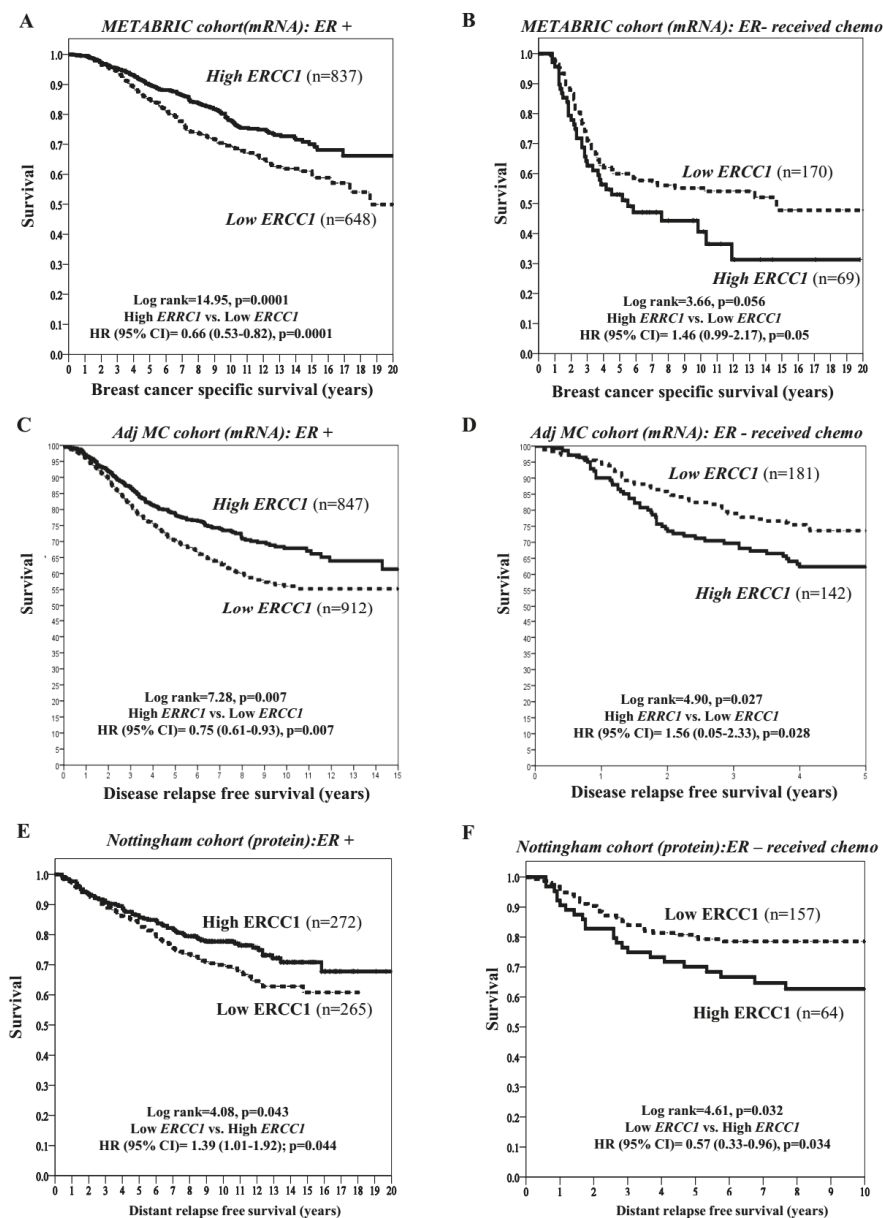
Table 1. Cont.

	ERCC1 mRNA Expression		p-Value	* p-Value(Adjusted)
	Low	High		
PAM 50 Her2				
Negative	799 (81.9%)	909 (93.9%)	$4.39 \times 10^{-16}$	<0.00001
Positive	177 (18.1%)	59 (6.1%)		
PAM 50 Basal				
Negative	751 (76.9%)	871 (90.0%)	$1.089 \times 10^{-14}$	<0.00001
Positive	225 (23.1%)	97 (10.0%)		
Integrative Molecular Clusters				
Int Clust 1	101 (10.3%)	35 (3.6%)	$1.163 \times 10^{-60}$	<0.00001
Int Clust 2	41 (4.2%)	30 (3.1%)		
Int Clust 3	78 (8.0%)	210 (21.6%)		
Int Clust 4	144 (14.8%)	187 (19.2%)		
Int Clust 5	139 (14.2%)	46 (4.7%)		
Int Clust 6	52 (5.3%)	33 (3.4%)		
Int Clust 7	74 (7.6%)	112 (11.5%)		
Int Clust 8	77 (7.9%)	221 (22.7%)		
Int Clust 9	108 (11.1%)	38 (3.9%)		
Int Clust 10	162 (16.6%)	62 (6.4%)		
Genufu Sub-Types				
ER-/Her-2-	104 (21.4%)	44 (8.8%)	$7.43 \times 10^{-37}$	<0.00001
ER+/Her-2- (high proliferation)	212 (43.7%)	148 (29.7%)		
ER+/Her-2- (low proliferation)	85 (17.5%)	281 (56.3%)		
Her-2 +	84 (17.3%)	26 (5.2%)		

\* p values were adjusted according to Benjamini-Hochberg method.

## 2.2. ERCC1 Transcript and Clinical Outcomes in Patients Receiving Adjuvant Therapy

In the ER+ METABRIC whole cohort, low ERCC1 transcript was associated with higher risk of death ( $p = 0.0001$ ) (Figure 1A). In patients who received endocrine therapy, similarly, low ERCC1 was associated with higher risk of death ( $p = 0.0001$ ) (Figure S2A). In addition, in the ER+ METABRIC cohort, we tested 188 DNA repair genes in a multivariate Cox proportional hazards model with backward stepwise exclusion and identified ERCC1 (among seven other genes) as an independent predictor for overall survival (OS) (Table S10). As chromosomal instability is a marker of chemo-sensitivity, we evaluated ERCC1 in ER- METABRIC patients who received chemotherapy. Low ERCC1 mRNA expression was associated with a decreased risk of death from BC ( $p = 0.05$ ) (Figure 1B). In a multivariate Cox regression analysis after controlling for confounders (such as endocrine therapy, chemotherapy and other validated prognostic factors (ER, PR, HER2, grade, stage, tumour size, TP53 mutation status, PAM50 molecular subtype and IntClust subclasses)), we confirmed that ERCC1 transcript was an independent prognostic factor for OS ( $p = 0.039$ ) and the interaction between ERCC1 and chemotherapy was also statistically significant ( $p = 0.020$ ) (Table 2). In ER- tumours, that received no chemotherapy, ERCC1 did not influence survival (Figure S2B).



**Figure 1.** ERCC1, adjuvant chemotherapy and survival. (A) Kaplan Meier curves showing BCSS (Breast cancer specific survival) based on ERCC1 mRNA expression in ER+ METABRIC cohort. (B) Kaplan Meier curves showing BCSS (Breast cancer specific survival) based on ERCC1 mRNA expression in ER– METABRIC cohort. (C) Kaplan Meier curves showing disease specific survival based on ERCC1 mRNA expression in ER+ Multicentre Adjuvant (Adj MC) cohort. (D) Kaplan Meier curves showing disease specific survival based on ERCC1 mRNA expression in ER– Adj MC cohort. (E) Kaplan Meier curves showing disease specific survival based on ERCC1 protein level in ER+ Adj MC cohort. (F) Kaplan Meier curves showing disease specific survival based on ERCC1 protein level in ER– Adj MC cohort.

**Table 2.** Multivariate Cox regression analysis for overall survival (OS) at 20 years in the METABRIC cohort.

Variables	HR	95.0% CI		p Value
		Lower	Upper	
<i>ERCC1</i> mRNA expression (+)	1.43	1.02	2.01	0.039 *
ER (+)	0.75	0.38	1.49	0.411
PR (+)	0.91	0.63	1.32	0.624
HER2 overexpression	0.82	0.36	1.85	0.63
TP53 mutation	1.81	1.24	2.63	0.002 *
Tumour Size (continuous)	1.01	1.01	1.02	0.001*
Lymph node (LN) stage				
Negative	1			
1–3 positive LNs	1.87	1.25	2.79	1.2 × 10 <sup>−6</sup> *
> 3 positive LNs	3.33	2.12	5.23	
Histological grade				
Low	1			
Intermediate	0.98	0.46	2.09	0.563
High	1.2	0.55	2.62	
PAM-50 subtypes				
PAM-50-LUM A	1	0.97	2.16	
PAM-50-LUM B	1.44	0.43	2.16	0.049 *
PAM-50-LUM HER2	0.96	0.5	3.04	
PAM-50-LUM Basal	1.24	1.17	4.56	
PAM-50-Normal like	2.31			
IntClust Members				
IntClust 1	1			
IntClust 2	1.28	0.59	2.8	
IntClust 3	0.56	0.25	1.24	
IntClust 4	0.8	0.4	1.59	
IntClust 5	2.47	0.94	6.53	0.189
IntClust 6	1.15	0.5	2.64	
IntClust 7	1.08	0.49	2.38	
IntClust 8	1.03	0.52	2.05	
IntClust 9	1.31	0.66	2.58	
IntClust 10	0.81	0.38	1.76	
Hormone therapy	0.64	0.43	0.96	0.031 *
Chemotherapy	0.93	0.62	1.41	0.741
Interaction term				
Hormone therapy * ER (IHC)	2.86	1.1	7.42	0.09
Interaction term				
Chemotherapy * <i>ERCC1</i>	2.11	1.23	3.95	0.020 *

\* significant *p* values.

We then validated in the Multicentre (MC)-Adjuvant cohort of 4640 patients. By using mean as cut-off, high and low *ERCC1* were observed in 49% (1460/2261) and 51% (1460/2379) of cases, respectively. ER and HER2 status were available for 3826 and 1727 cases; respectively. About 59% (2268/3826), 41% (1558/3826) and 26% (446/1727) of cases were ER+, ER− and HER2+, respectively (Tables S3 and S4). Similar to the METABRIC data, in ER+ tumours, low levels of *ERCC1* was associated with an increased distant relapse DRR compared to high levels of *ERCC1* [*p* = 0.007] (Figure 1C). However, in ER− tumours that received adjuvant chemotherapy, low *ERCC1* was associated with a reduced DRR compared to high *ERCC1* (*p* = 0.001) (Figure 1D). Multivariable Cox regression models confirmed that

the low *ERCC1* transcript is a poor prognostic factor for DRR after controlling with Adjuvant! Online (AOL) ( $p = 0.047$ ) and 72-proliferation-gene-signatures ( $p < 0.0001$ ).

### 2.3. *ERCC1* Protein, Clinicopathological Features and Outcomes

Using median as the cut off ( $H$ -score  $\geq 130$ ), we observed *ERCC1* nuclear protein expression in 439/991 (44.3%) of breast tumours, and 55.7% (552/991) were negative for *ERCC1* expression. As shown in (Table S11), low nuclear *ERCC1* level was significantly associated with aggressive phenotypes, including high grade, no special histological type (NST), ER-, basal-like phenotype and triple negative tumours, as well as loss of other DNA repair biomarkers (all adjusted  $p \leq 0.01$ ). In ER+ tumours, low *ERCC1* protein was linked to poor disease relapse free survival ( $p = 0.044$ ) (Figure 1E). On the other hand, in ER- tumours that received chemotherapy, low *ERCC1* protein was linked to improved disease relapse free survival ( $p = 0.034$ ) (Figure 1F). Multivariable Cox regression analysis controlling for chemotherapy, endocrine therapy and other validated prognostic factors (stage, grade, size, ER, PR, HER2 and BCL2), showed that *ERCC1* protein expression was an independent prognostic factor for OS ( $p = 0.035$ ) and that the interaction between *ERCC1* protein expression and adjuvant chemotherapy was statistically significant ( $p = 0.022$ ) (Table S12).

### 2.4. *ERCC1* and Pathological Complete Response (pCR) to Neoadjuvant Chemotherapy

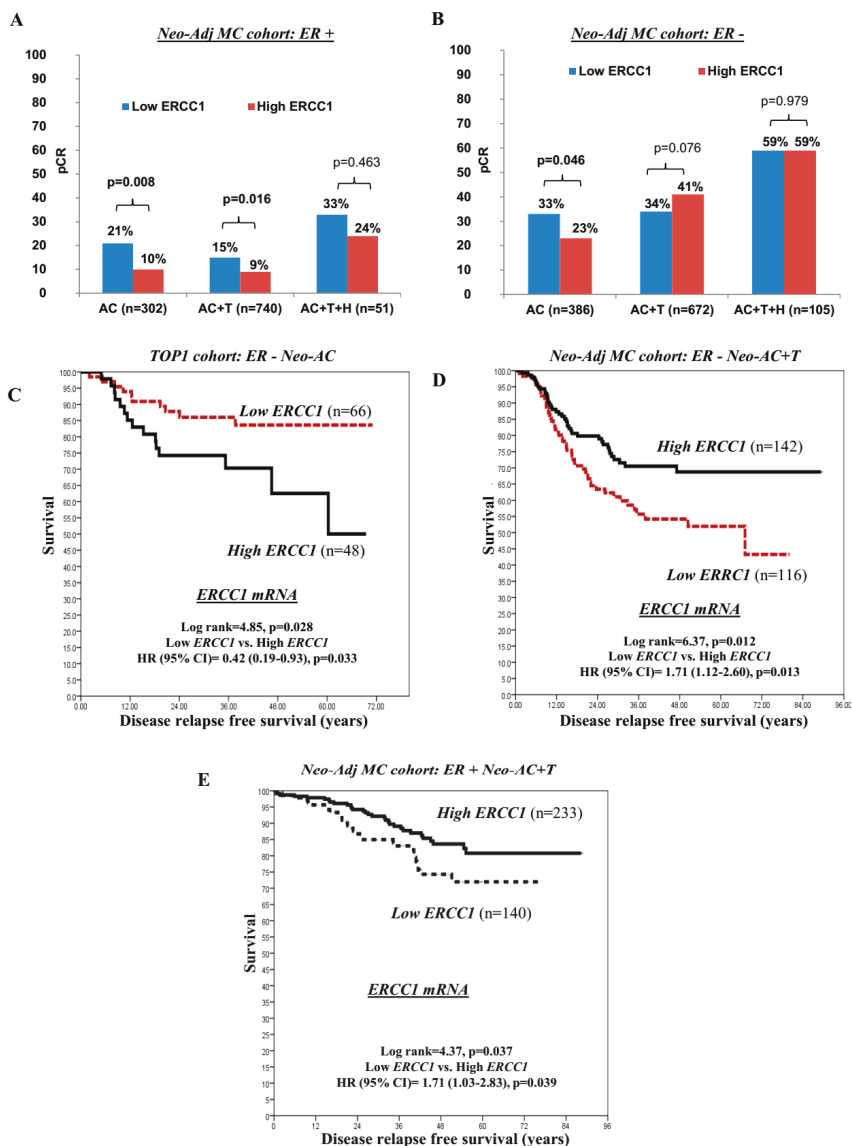
Neoadjuvant chemotherapy (pre-operative) is an established approach in locally advanced breast cancers (LABC). Although current evidence suggests that patients who achieve pCR have a better long-term clinical outcome [27,28], the development of a predictive biomarker of pCR remains a high priority. We therefore evaluated *ERCC1* transcripts in a multiple centre cohort of 2345 LABC patients who received neoadjuvant anthracycline based combination (Neo-Adj) AC-chemotherapy (CT) + or - T with or without Herceptin (+ or - H), including multiple clinical trials sub cohorts (MC-Neo-Adjuvant cohort). The majority of patients (60%; 1413/2345) had received Neo-Adj AC-CT+T (taxane) whereas 29% (689/2345) and 10% (243/2345) of patients had received Neo-Adj AC-CT alone and AC-CT+T+H; respectively. About 52% of cases were ER- (1163/2256) whereas 48% (1093/2345) and 24% (518/2163) were ER+ and HER2+, respectively. Low and high *ERCC1* transcript expressions were observed in 48% (1133/2345) and 52% (1212/2345) of cases, respectively. Out of the 2345 patients, 596 (25%) patients had achieved pCR. Low *ERCC1* transcript expression was associated with an increased proportion of patients achieving pCR (333 (29%) of 1133 patients) compared with high *ERCC1* transcript expression (263 (22%) of 1212 patients; OR (95% CI): 1.50 (1.25–1.81,  $p < 0.0001$ ).

In ER+ patients, low *ERCC1* transcript expression was also associated with a higher proportion of patients achieving pCR (80 (18%) of 442 patients) compared with high *ERCC1* transcript expression (64 (10%) of 651 patients; OR (95% CI): 2.03 (1.42–2.89),  $p < 0.0001$ ) especially in ER+ patients who received either Neo-Adj AC-CT alone (21% (29/136) versus 10% (17/166); OR (95% CI): 2.38 (1.24–4.55),  $p = 0.008$ ) or Neo-Adj ACT-CT+T (15% (41/276) versus 9% (42/464); OR (95% CI): 1.75 (1.11–2.77),  $p = 0.016$ ) (Figure 2A).

In ER- patients who received Neo-Adj AC-CT alone, low *ERCC1* transcript expression was also associated with a higher proportion of patients achieving pCR (74 (33%) of 227 patients) compared with high *ERCC1* transcript expression (37 (23%) of 159 patients (OR (95% CI): 1.59 (1.01–2.53),  $p = 0.046$ ) (Figure 2B). We validated this observation in the TOP1 trial cohort of ER-negative tumours where patients received anthracycline (epirubicin) monotherapy only. Low *ERCC1* transcript expression was associated with an increased proportion of patients achieving pCR (12 (21.4%) of 56 patients) compared with high *ERCC1* transcript expression (4 (6.9%) of 58 patients; OR (95% CI): 3.683 (1.11–12.20),  $p = 0.026$ ). Moreover, in the TOP1 cohort, low *ERCC1* transcript expression was associated with 58% lower relapse risk compared to high *ERCC1*, (HR (95% CI): 0.42 (0.19–0.93);  $p = 0.033$ ) (Figure 2C). For additional validation at protein level, we investigated the effect of *ERCC1* protein on pCR in a series of 120 LABC patients who received Neo-Adj AC-CT alone. 19/120 (16%) patients achieved pCR in this cohort. Low *ERCC1* protein expression was associated with an increased proportion of patients



achieving a pCR (16 (26%) of 62 patients) compared with high ERCC1 protein expression (3 (5%) of 57 patients; OR (95% CI): 6.25 (1.89–22.73,  $p = 0.002$ ).



**Figure 2.** ERCC1 and neoadjuvant chemotherapy. (A) Pathological complete response (pCR) based on ERCC1 mRNA expression in ER+ tumours (neoadjuvant anthracycline based (Neo-Adj) MC cohort) that received neoadjuvant AC or AC+T or AC+T+H chemotherapy. (B) Pathological complete response (pCR) based on ERCC1 mRNA expression in ER– tumours (Neo-Adj MC cohort) who received neoadjuvant AC or AC+T or AC+T+H chemotherapy. (C) Disease free survival based on ERCC1 mRNA expression in TOP1 cohort patients who received neoadjuvant AC chemotherapy. (D) Disease free survival based in ER– Neo-Adj MC cohort who received neoadjuvant AC+T chemotherapy. (E) Disease free survival based in ER+ Neo-Adj MC cohort who received neoadjuvant AC+T chemotherapy.

On the other hand, in ER− patients who received Neo-Adj ACT+T, high *ERCC1* transcript expression was associated with a higher proportion of patients achieving a pCR (142 (41%) of 349 patients) compared with low *ERCC1* transcript expression (110 (34%) of 323 patients; OR (95% CI): 1.33 (0.97–1.82),  $p = 0.076$ ) (Figure 2B). In addition, in ER− patients who received pre-operative Neo-Adj ACT+T, low *ERCC1* had higher relapse risk compared to high *ERCC1* (HR (95% CI): 1.71 (1.12–2.60);  $p = 0.013$ ) (Figure 2D). Similarly, in ER+ patients also who received Neo-Adj ACT+T, low *ERCC1* had higher relapse risk compared to high *ERCC1* (hazard ratio (HR) (95% CI) = 1.71 (1.03–2.83),  $p = 0.039$ ) (Figure 2E).

Taken together, the data provides compelling evidence that *ERCC1* has prognostic significance in ER+ BCs and predict response to chemotherapy in ER− BCs.

### 3. Discussion

Although the efficacy of DNA damaging chemotherapy (such as anthracyclines) is influenced by impaired DNA repair capacity, evolving evidence also suggests that mitotic spindle poisons (such as taxanes) are more effective in low grade chromosomally stable tumours. Therefore the development of robust DNA repair based biomarkers is highly desirable. *ERCC1* is non-catalytic but partners with XPF endonuclease to form the *ERCC1*–XPF heterodimer which processes abnormal DNA repair intermediates generated during NER, double strand breaks (DSB) repair and Interstrand Cross Link (ICL) repair [29]. Given the key role for *ERCC1* in genomic integrity, we hypothesized a role for *ERCC1* in breast cancer pathogenesis and response to therapy. In the current study we show that *ERCC1* transcript expression was independently associated with OS in the METABRIC cohort. In ER+ tumours, low *ERCC1* transcript or protein level was associated with increased distant relapse risk (DRR). In ER− tumours, low *ERCC1* transcript or protein level was linked to decreased DRR, especially in patients who received anthracycline chemotherapy. In LABC patients who received neoadjuvant anthracycline, low *ERCC1* transcript was associated with higher pCR (pathological complete response) and decreased DRR. However, in patients with ER− tumours who received additional neoadjuvant taxane, high *ERCC1* transcript was associated with a higher pCR and decreased DRR. High *ERCC1* transcript was also linked to decreased DRR in ER+ LABC that received additional neoadjuvant taxane. Taken together, the data presented here provides comprehensive clinical evidence that *ERCC1* is a predictor of anthracycline resistance and taxane sensitivity in breast cancers. *ERCC1* based stratification could be an attractive strategy in breast cancers.

Studies exploring biomarkers of response to anthracycline therapy have been limited in breast cancers. Previous smaller studies suggest that Ki67, HER1-3 expression, TOP2A and HER-2 are potential markers of anthracycline benefit [30]. Ch17CEP, a CIN marker, was also previously shown to predict benefit from anthracycline adjuvant chemotherapy in a prospective clinical trial [8]. *ERCC1* is a critical factor for CIN. To the best of our knowledge, the data shown here represents the first comprehensive evidence that *ERCC1* status influences potential benefitting from anthracycline chemotherapy. The role of *ERCC1* in breast cancer pathogenesis is emerging. *ERCC1* polymorphism may be associated with increased breast cancer risk [31]. A previous, small study suggested that *ERCC1* protein levels may be low in triple negative breast cancers (TNBCs) [32,33]. In a study of fifty two TNBCs, *ERCC1* positivity was associated with shorter progression free survival and poor response to neoadjuvant chemotherapy [32]. In addition, *ERCC1* genetic polymorphism also appeared to associate with pCR in patients receiving neoadjuvant anthracycline chemotherapy [34]. Gay-Beylille et al. recently also demonstrated that *ERCC1* expression is induced in tumours that receive anthracycline based neoadjuvant chemotherapy [35]. Previous clinical studies have evaluated the predictive significance of *ERCC1* for response to platinum chemotherapy, in various solid tumours (lung, colorectal, head and neck, gastric and bladder cancers [36–39]). However, a major limitation has been the use of relatively non-specific *ERCC1* antibodies for immunohistochemistry in previous studies, including in a large lung cancer clinical trial [40]. In the current study we utilised a recently generated and highly specific mouse monoclonal antibody (clone 4F9) [41], which further strengthens our clinical data. Our study not only

concur with previous studies showing a link between ERCC1 overexpression and chemoresistance but also provides additional insight suggesting that ERCC1 may also be involved in the emergence of aggressive breast cancer phenotypes. However, a limitation to our study is that it is predominantly retrospective. Prospective studies would be required to confirm our findings.

Currently there is no established predictive biomarker of response to taxane therapy. Previous studies suggest that HER2, Ki67, class III  $\beta$  tubulin expression may influence taxane response [10]. A novel observation in the current study is that ERCC1 was also shown to influence whether taxane chemotherapy was beneficial. Our data concurs with previous evidence demonstrating taxane benefit in low grade, chromosomally stable tumours [9,10]. However, further prospective studies would be required to confirm our initial findings.

Taken together, the data would support further development of ERCC1 as a biomarker of response to chemotherapy in breast cancer.

#### 4. Patients and Methods

##### 4.1. Study Design and Cohorts

Study design, the patient cohorts which included 11,096 BCs and their demographics are summarized in the consort flow diagram (Figure 3), also in Supplementary Methods and Tables.

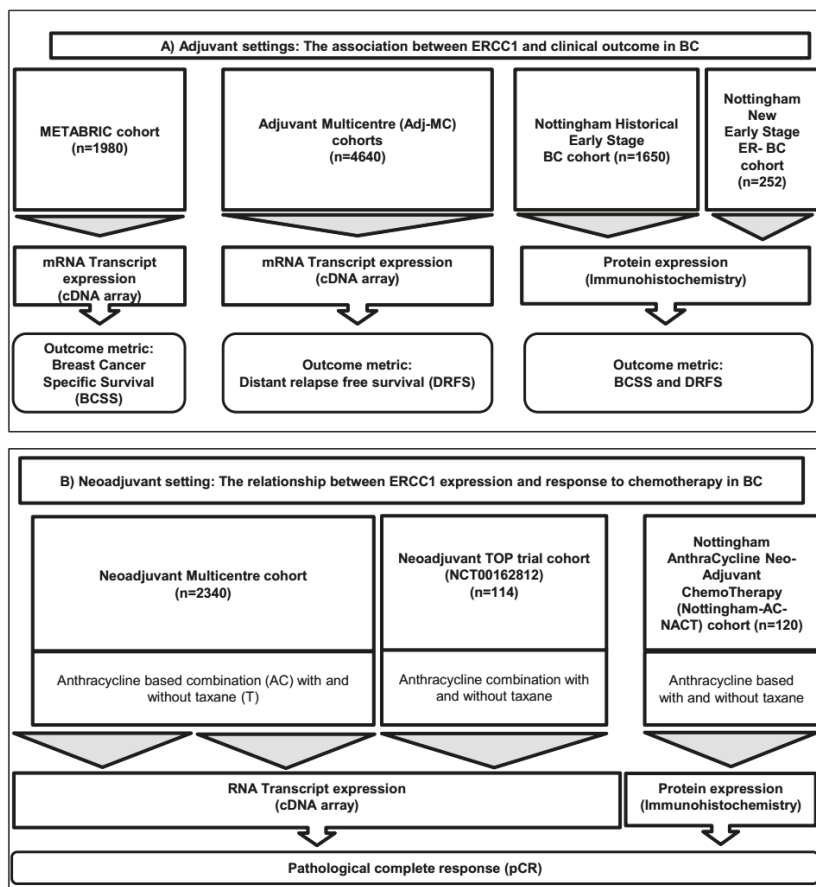


Figure 3. Consort diagram summarizing patient cohorts investigated in the current study.

## 4.2. Outcome Measurements and Patient Cohorts:

### 4.2.1. ERCC1 Transcript Expression Analysis

I The association of 188 DNA repair genes and prognosis (overall survival; OS) analysis:

Cohort (1): METABRIC cohort (Molecular Taxonomy of BC International Consortium)

Patient demographics are summarized in Supplementary Table S1. We investigated the association of 188 DNA repair genes (Table S2) with OS in the METABRIC cohort (METABRIC  $n = 1980$ ; median follow-up time in years (MFUT) (inter-quantile range (IQR): 9.1 (5.2–12.9)). Univariate Cox regression analysis was used in SPSS (Version 20, Chicago, IL, USA) and the Benjamini and Hochberg False Discovery Rate calculation (BH FDR) was applied to account for multiple comparisons. After definition of factors that were associated with OS after BH FDR correction, multivariate Cox proportional hazards models (with backward stepwise exclusion of these factors, using a criterion of  $p < 0.05$  for retention of factors in the model) were used to identify factors that were independently associated with OS. The statistical significance of the model was assessed based on the likelihood ratio test. The proportional hazards assumption was tested using both standard log–log plots and by generating Kaplan–Meier survival estimate curves, and observing that the curves did not intersect with each other. Hazard ratios (HRs) for death risk and 95% confidence intervals were calculated from the Cox proportional hazards analysis.

II The association of distant relapse risk (DRR) and ERCC1 transcript after receiving systemic adjuvant therapy (Adj-T):

Cohort (2): Multicentre (MC)-Adjuvant cohort ( $n = 4640$ )

The association between ERCC1 mRNA expression and DRR and its relationship with the received systemic Adj-T were tested in 4640 patients with early stage BC, retrieved from 21 gene expression databases (see Supplementary Tables S3 and S4). ERCC1 gene expression data of each database were converted to a common scale (median equal to 0 and standard deviation equal to 1) in order to merge all of the study data that used the same platform and to create combined cohorts. Then the data was median centred for each gene, whereby the median of each gene was 0. Databases using the same platform were merged and the median expression was calculated. The median expression of ERCC1 transcription for each platform was calculated and values equal to or higher than the median coded as +1 (overexpression). Values of less than the median were coded 0 or low ERCC1. Distal relapse free survival follow up data were available for 3171 patients with 967 events (MFUT (IQR): 5.5 (3.0–8.7)). The systemic Adj-T information was available for 2276 patients: 45% of patients were naïve to systemic Adj-T, whereas 49% had received Adj-endocrine therapy and 27% had received chemotherapy. Herceptin had been offered to 156 (7%) of HER2 + patients.

III The association between ERCC1 and pathological complete response rate (pCR) analysis after receiving neoadjuvant anthracycline based combination chemotherapy (Neo-Adj-ACT)

Cohort (3): Multicentre (MC) Neo-Adjuvant cohort ( $n = 2340$ )

Demographics summarized in Supplementary Table S5. The association between ERCC1 mRNA expression and pCR was evaluated in 2340 patients retrieved from 15 gene expression databases that received pre-operative anthracycline based combination (AC) with (+) or without (–) taxane (T). Out of 2345 patients, 689 patients (29%) has received Neo-Adjuvant anthracycline based combination chemotherapy (Neo-Adj-ACT) alone; 1413 patients (60%) received Neo-Adj-ACT with Taxane (ACT + T) and 243 patients received Neo-Adj-ACT + T + Herceptin (ACT + T + H).

Cohort (4): Neoadjuvant TOP trial cohort (NCT00162812), in which patients with oestrogen receptor (ER)-negative tumours were treated with anthracycline (epirubicin) monotherapy. Demographics summarized in Supplementary Table S6.

### 4.2.2. Protein Expression Association Analysis

Immunohistochemical evaluation of ERCC1 protein expression was performed in three cohorts of patients who treated at a single centre (Nottingham University Hospital (NUH)). We utilised a recently

characterised highly specific anti-ERCC1 mouse monoclonal antibody (clone 4F9, Dako Ltd., Cheshire, UK) [18]. We confirmed the specificity of clone 4F9, using Western Blots in breast cancer (SKBR3, T47D and MDA-MB-231) and ovarian cancer (A2780 and A2780cis) cell lines (Supplementary Figure S1A). Tissue culture and Western blot analyses is described in Supplementary Methods.

#### 4.3. Adjuvant Setting

Cohort (5): NUH- early stage breast cancer (NUH-ESBC): The study was performed in a well characterised consecutive series of 1650 patients with primary invasive breast carcinomas who were diagnosed between 1986 and 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series [42] (Nottingham historical early stage cohort (NUH-ESBC); MFUT (IQR): 13.4 (10.3–16.42)). Demographics are summarized in Supplementary Table S7. Supplementary Methods provide a detailed description on Tissue Microarrays (TMAs) and immunohistochemistry (IHC) evaluations (Table S8). The association between the ERCC1 protein expression with clinicopathological parameters and DRR were analysed in this cohort.

Cohort (6): (Nottingham ER-negative series). A series of 252 ER negative invasive breast carcinomas diagnosed and managed at the Nottingham University Hospitals (NUH) Trust between 1999 and 2007. All patients were primarily treated with surgery, followed by adjuvant radiotherapy and anthracycline based combination chemotherapy [43]. Demographics are shown in Supplementary Table S9.

#### 4.4. Neo-Adjuvant Setting

Cohort (7): Nottingham anthracycline based neo-adjuvant chemotherapy cohort (Nottingham AC-NACT;  $n = 120$ ) consisting of pre-chemotherapy core biopsies from 120 female patients with locally-advanced primary BC treated with neo-adjuvant (Neo-Adj) anthracycline-based combination chemotherapy (AC-CT) (Neo-Adj-AC-CT) treated at NUH between 1996 and 2012 [42].

All patients completed written informed consented, as per hospital standard of care, for excess tumour tissue to be used in research. The study was approved by the Institutional Review Board or Independent Ethics Committee and the Hospital Research and Innovations Department at all participating sites. Tumour Marker Prognostic Studies (REMARK) criteria, as recommended by McShane et al. [44] were followed throughout this study.

#### 4.5. Power Analysis

A retrospective power analysis was conducted to determine the confidence in the calculated hazard ratio and associated  $p$  value for 10 year survival and to ascertain how applicable the result would be to a global population. Power of study was determined using PASS (NCSS, version 13, USA).

#### 4.6. Statistical Analysis

Statistical analyses were performed using STATISTICA (Stat Soft Ltd., Tulsa, OK, USA) and SPSS (version 17, Chicago, IL, USA) by the authors who were blinded to the clinical data. Where appropriate, Pearson's chi-squared, student's t-test and ANOVA tests were used. Positivity for ERCC1 protein both pre- and post-chemotherapy was calculated and compared using McNemar's test. Cumulative survival probabilities and 10-year BCSS and DFS were estimated using the univariate Cox proportional hazards models and the Kaplan–Meier plot method where appropriate, and differences between survival rates were tested for significance using the log-rank test. Multivariable analysis for survival was performed using the Cox proportional hazard model. The proportional hazards assumption was tested using standard log–log plots. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable. All tests were two-sided with a 95% CI, and a  $p$  value  $< 0.05$  was considered to be indicative of statistical significance. The interaction between ERCC1 and chemotherapy was tested in the Cox proportional hazard model. For multiple comparisons,  $p$  values were adjusted according to Benjamini–Hochberg method [45]. Tumor Marker Prognostic Studies (REMARK) criteria,

recommended by McShane et al. [46], were followed throughout this study. Ethical approval was obtained from the Nottingham Research Ethics Committee (C202313).

## 5. Conclusions

ERCC1 is non-catalytic but partners with XPF endonuclease to form the ERCC1–XPF heterodimer which processes abnormal DNA repair intermediates generated during NER, DSB repair and ICL repair. We provide the first comprehensive clinical evidence that ERCC1 is a key predictor of chemotherapy response in patients with breast cancer who receive adjuvant or neoadjuvant chemotherapy. Importantly, the clinical study suggests that ERCC1 based stratification is feasible in BC patients who receive anthracycline and/or taxane chemotherapy.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/11/8/1149/s1>, Table S1: Clinicopathological characteristics in the METABRIC cohort, Table S2: List of DNA repair genes tested in the METABRIC cohort, Table S3: Cohort ID and gene expression platform Multicentre (MC)-Adjuvant cohort (n=4640), Table S4: Demographics of Multicentre (MC)-Adjuvant cohort (n=4640), Table S5: Multicentre (MC) Neo-Adjuvant cohort (n= 2345), Table S6: Demographics in TOP trial cohort, Table S7: Clinicopathological characteristics of Nottingham historical early, TableS8: Table of antibodies and optimisation conditions used to immunohistochemically profile the Nottingham University Hospitals based cohorts. Detailed below are: Antigens, primary antibodies, clone, source, optimal dilution and scoring system, used for each immunohistochemical marker stage cohort (NUH-ESBC), Table S9: Clinicopathological characteristics of ER- cohort, Table S10: Multivariate backward step-wise Cox analysis for DNA repair genes, associated with Overall Survival (OS) in ER+ METABRIC cohort, Table S11: Clinicopathological significance of ERCC1 protein expression in breast cancers, Table S12: Multivariate Cox regression analysis for breast cancer specific survival (BCSS) at 20 years follow up in Nottingham series including interaction terms.

**Author Contributions:** Study design, T.M.A.A.-F. and S.M.; data collection, data analyses, data interpretation, drafting the manuscript and approving the final version, T.M.A.A.-F., S.M., R.A., M.S., P.M.M., K.A.M., G.B., A.R.G., E.A.R. and S.Y.T.C.

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Article

# Dasatinib Treatment Increases Sensitivity to c-Met Inhibition in Triple-Negative Breast Cancer Cells

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**Abstract:** In pre-clinical studies, triple-negative breast cancer (TNBC) cells have demonstrated sensitivity to the multi-targeted kinase inhibitor dasatinib; however, clinical trials with single-agent dasatinib showed limited efficacy in unselected populations of breast cancer, including TNBC. To study potential mechanisms of resistance to dasatinib in TNBC, we established a cell line model of acquired dasatinib resistance (231-DasB). Following an approximately three-month exposure to incrementally increasing concentrations of dasatinib (200 nM to 500 nM) dasatinib, 231-DasB cells were resistant to the agent with a dasatinib IC<sub>50</sub> value greater than 5 µM compared to 0.04 ± 0.001 µM in the parental MDA-MB-231 cells. 231-DasB cells also showed resistance (2.2-fold) to the Src kinase inhibitor PD180970. Treatment of 231-DasB cells with dasatinib did not inhibit phosphorylation of Src kinase. The 231-DasB cells also had significantly increased levels of p-Met compared to the parental MDA-MB-231 cells, as measured by luminex, and resistant cells demonstrated a significant increase in sensitivity to the c-Met inhibitor, Cpda, with an IC<sub>50</sub> value of 1.4 ± 0.5 µM compared to an IC<sub>50</sub> of 6.8 ± 0.2 µM in the parental MDA-MB-231 cells. Treatment with Cpda decreased p-Met and p-Src in both 231-DasB and MDA-MB-231 cells. Combined treatment with dasatinib and Cpda significantly inhibited the growth of MDA-MB-231 parental cells and prevented the emergence of dasatinib resistance. If these in vitro findings can be extrapolated to human cancer treatment, combined treatment with dasatinib and a c-Met inhibitor may block the development of acquired resistance and improve response rates to dasatinib treatment in TNBC.

**Keywords:** Src kinase; basal-like breast cancer; cMet

## 1. Introduction

Triple-negative breast cancer (TNBC) lacks expression of estrogen receptor (ER) and progesterone receptor (PR) and exhibits overexpression of human epidermal growth factor receptor 2 (HER2). It accounts for approximately 15% of all breast cancer cases and patients with this form demonstrate higher rates of recurrence and shorter disease progression than those with luminal or HER2-positive

tumours [1]. Treatment of TNBC predominantly relies on the use of cytotoxic chemotherapies due to a lack of proven molecular targets [2].

We have previously shown that Proto-oncogene tyrosine-protein kinase Src (Src kinase) is frequently expressed in TNBC and may be a rational therapeutic target for TNBC [3]. Src is a proto-oncogene and a member of the Src family kinases (SFKs). SFKs are non-membrane-bound tyrosine kinases consisting of nine members. Src, Yes, Fyn and Fgr belong to the Src A family subtype. Lck, Hck, Blk and Lyn belong to Src B family subtype, with Frk in its own subfamily [4]. The SFKs display high levels of homology to one another and are involved in propagating downstream cell signalling to effect cellular biological functions including cell proliferation, cell migration, angiogenesis and cell survival [5–7].

Dasatinib is a multi-targeted tyrosine kinase inhibitor whose targets include BCR/Abl and Src family kinases. Pre-clinical studies suggested that basal-like TNBC cell lines show higher sensitivity to dasatinib than luminal cell lines, supporting dasatinib as a potential targeted treatment for TNBC [3,8,9]. Additionally, when TNBC was further sub-classified into six molecular types, cell line models of the mesenchymal and mesenchymal stem-like group showed greater sensitivity to dasatinib than other cell line models [3,9,10]. However, in a phase II clinical trial, dasatinib showed limited single-agent activity in patients with metastatic breast cancer [11] or in patients with locally advanced or metastatic TNBC [12].

Several potential predictive biomarkers of response to dasatinib treatment have been identified [13], and a prospective phase II trial was designed to test three predictive gene signatures, whereby patients with metastatic breast cancer whose metastatic tumour biopsies were positive for one of the gene signatures were treated with dasatinib [13]. However, no significant clinical benefit was observed despite selecting patients having the highest scores for the three predictive signatures.

Thus, despite promising activity in preclinical studies, dasatinib has so far failed to produce clinical benefits in TNBC patients. One of the limitations of the preclinical studies that showed activity in TNBC cells, including our own [3], is that they were limited to short-term proliferation assays. Therefore, we examined the effects of longer exposure to dasatinib on TNBC cells that are highly sensitive to dasatinib in short-term proliferation assays. The overall aim was to identify novel combinatorial approaches that may block the development of acquired resistance to dasatinib and improve response rates to dasatinib treatment in TNBC.

## 2. Materials and Methods

### 2.1. Cell Lines and Reagents

MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC). 231-DasB was developed by continuous exposure to dasatinib (Sequoia Research Products, Berkshire, UK) with a starting concentration of 200 nM, increasing incrementally to a maximum of 500 nM dasatinib over a period of 13 weeks. Untreated (parental) MDA-MB-231 cells were cultured in a similar manner in the absence of drug to create an aged-matched control. Both cell lines were cultured in RPMI 1640 with 10% Foetal Bovine Serum, plus 10 nM dasatinib for 231DasB. Stock solutions (10 mM) of PD180970 (Merck, Dublin, Ireland), elacridar (Sequoia Research Products, Berkshire, UK) and Compound A (CpdA, Amgen, Thousand Oaks, CA, USA) were prepared in DMSO.

### 2.2. Proliferation Assays

Proliferation was measured in triplicate biological assays using an acid phosphatase assay or viable cell count. For acid phosphatase assays,  $1 \times 10^3$  cells/well were seeded in 96-well plates. Plates were incubated overnight at 37 °C followed by the addition of the drug at the appropriate concentrations and incubated for a further five days until wells were 80–90% confluent. All media were removed and the wells were washed once with phosphate buffered solution (PBS). Paranitrophenol phosphate (PNP) substrate (Sigma-Aldrich, Dublin, Ireland) (10 mM of PNP in sodium acetate buffer, pH 5.5) was

added to each well and incubated at 37 °C for 1 h. Fifty microliters of 1 M NaOH were added and the absorbance was read at 405 nM (reference: 620 nM). For viable cell counts, cells were trypsinized and combined in a 1:1 ratio with Viacount Flex reagent and counted using a Guava EasyCyte (Merck, Dublin, Ireland).

### 2.3. Doubling Time Assays

Next  $2 \times 10^3$  cells were seeded in 24-well plates in 1 mL of serum-containing medium. Fresh medium (control) or drug-containing medium was added to the cells. Assays were conducted in triplicate. Duplicate wells per cell line for each condition (control and drug treatments) were seeded for each time point: day 0, day 4, and day 7. Cell counts were measured using the Guava Viacount method. Doubling times were calculated between days 4 and 7 using the formula

$$\text{Doubling time (hours)} = 24 \times \frac{\log 2x(Tt - T0)}{(\log Nt - \log N0)},$$

where Tt is the end time point and T0 is the beginning time point (days), which in this case were 7 and 4, respectively, and N is the average cell count on each day.

### 2.4. Short-Term Resistance Assay

For the short-term resistance assays,  $1.5 \times 10^4$  MDA-MB-231 cells per well were seeded in two identical 12-well plates (Plate-1 and Plate-2) in 1 mL of medium with 10% foetal bovine serum (day 1) and allowed to adhere overnight. The cells were treated with 2.5  $\mu$ M compound A and/or 50 nM dasatinib. In addition, in plate 1, three wells were untreated as a control. In plate 2, the cells were similarly treated with 2.5  $\mu$ M compound A and/or 50 nM dasatinib. Treatment was repeated twice weekly. After seven days, when the untreated control cells achieved confluency, the medium was removed from the cells in plate 1 and the cells were fixed with 3:1, v:v, methanol:acetic acid, (1 min), then washed with PBS and stained with 0.05%, w:v, crystal violet (5 min). The cells were allowed to air dry overnight, the crystal violet was eluted in 10% acetic acid and absorbance was measured at 590 nM. After 21 days, when the dasatinib-treated cells achieved confluency, cells in plate 2 were fixed and stained as for plate 1.

### 2.5. Invasion/Migration Assays

Invasion and migration assays were carried out using  $5 \times 10^4$  cells in Matrigel (Corning)-coated 24-well invasion inserts (BD Biosciences) for invasion assays and uncoated inserts for migration assays. Cells were seeded in reduced serum medium (5% foetal calf serum in RPMI-1640) and incubated for 6 h to allow cell attachment, and then treated with 100 nM dasatinib for 18 h. Cells were stained with crystal violet and the number of invading/migrating cells was estimated by counting 10 fields of view at 200 $\times$  magnification. The average count was multiplied by the conversion factor 140 (growth area of membrane divided by field of view area, viewed at 200 $\times$  magnification) to determine the total number of invading/migrating cells. Invasion and migration assays were carried out in triplicate.

### 2.6. Dasatinib Accumulation Assays

Cells were seeded at the specified numbers in T25-cm<sup>2</sup> cell culture flasks (Thermo Fisher, Dublin, Ireland). After 24 h, the cell culture medium was removed and cells were treated with medium (control) or medium containing 2  $\mu$ M dasatinib for 2 h. Non-adherent cells were collected and combined with adherent cells after trypsinisation, then centrifuged at 300 g for 3 min. The supernatant was removed and the cells were resuspended in 1 mL of ice-cold PBS. Fifty microliters of the cell suspension were removed for cell counting using the Guava Viacount method. The cell suspension was centrifuged as before, and the supernatant was removed. The cell pellet was stored at -20 °C.

Quantification of the mass of dasatinib present in the cells collected was performed using liquid chromatography tandem mass spectrometry (LC-MS/MS), as previously described [14]. Briefly, the drug was extracted using a liquid-liquid extraction procedure. One hundred microliters of 500 ng/mL lapatinib were added to an extraction tube (internal standard), along with 200  $\mu$ L of 1 M Ammonium Formate pH 3.5 buffer and 1.6 mL of extraction solvent tert-Butyl Methyl Ether (tBME)/ acetonitrile (ACN) (3:1 v:v). The extraction tubes were vortexed and mixed on a blood tube mixer for 15 min. The samples were centrifuged at 6500 g for 5 min; the organic layer was removed and 1.1 mL of solvent was transferred to conical bottomed glass LC autosampler vials (Sigma-Aldrich, Dublin, Ireland). The vials were evaporated to dryness using a Genevac EZ-2 (Ipswich, UK) evaporator at ambient temperature, without light. The samples were reconstituted in 40  $\mu$ L of acetonitrile with 20  $\mu$ L injected automatically by the autosampler. The LC-MS was run in isocratic mobile phase (54% ACN:10 mM ammonium formate, pH 4) on a Hyperclone BDS C18 column, in multi-reaction monitoring (MRM) positive ion mode. Analysis was performed using MRM mode with the following transitions: m/z 581  $\rightarrow$  m/z 365 for lapatinib, and m/z 488  $\rightarrow$  m/z (231 and 401) for dasatinib, with a dwell time of 200 ms. Quantification was based on the integrated peak area determined by the Masshunter Quantification Analysis software, which quantitates the peak areas of the MRM transitions of each analyte. Results are reported as mean and standard deviation (SD) of the mass per million cells in triplicate flasks.

### 2.7. Protein Extraction and Western Blotting

RIPA buffer with 1 $\times$  protease inhibitors, 2 mM PMSF and 1 mM sodium orthovanadate (Sigma-Aldrich) was added to cells and incubated on ice for 20 min. Following centrifugation at 10,000 rpm for 10 min at 4  $^{\circ}$ C, the resulting lysate was stored at  $-80$   $^{\circ}$ C. Protein quantification was performed using the bicinchoninic acid (BCA) assay (Pierce). Thirty micrograms of protein in sample buffer were heated to 95  $^{\circ}$ C for 5 min and proteins were separated on 7.5% gels (Lonza) or 4–12% gels (Thermo-Fischer, Dublin, Ireland). The protein was transferred to a nitrocellulose membrane. The membrane was blocked with a blocking solution (PBS + 0.1% Tween + 5% skimmed milk powder (BioRad, Dublin, Ireland) or a 1 $\times$  NET solution buffer (0.5 M NaCl, 0.05 M EDTA, 0.1 M Tris pH 7.8) at room temperature for 1 h, then incubated overnight at 4  $^{\circ}$ C in a blocking solution with a 1:1000 antibody dilution of total Src, c-Met, p-Met Y1234/1235 (Cell Signalling Technology, Leiden, Netherlands) or 1:500 p-Src Y419 (Merck-Millipore). The membrane was washed three times with PBS-Tween or 1 $\times$  NET, then incubated at room temperature with anti-mouse secondary antibody (Sigma-Aldrich) at 1:1000 dilution or anti-rabbit secondary antibody (Sigma-Aldrich) at a 1:1000 dilution in blocking solution for 1 h. The membrane was washed three times with PBS-Tween/1 $\times$  NET followed by one PBS wash. Detection was performed using Luminol (Santa Cruz Biotechnology, Heidelberg, Germany).

### 2.8. Luminex Magnetic Bead Assays

Magnetic bead assays were performed on the Luminex<sup>®</sup> MagPix<sup>®</sup> System (Merck Millipore (80-073), Dublin, Ireland) using Milliplex Map Phospho Mitogenesis RTK Magnetic Bead 7-Plex Kit (Merck Millipore 48-672 Mag) and Milliplex Map phospho Human Src Family Kinase Magnetic Bead 8-Plex kit (Merck Millipore 48-650 Mag). Protein extractions were prepared as described above. Protein (1–10  $\mu$ g) was diluted in appropriate volume of assay buffer (final volume: 25  $\mu$ L/well) and the assay was performed as per the manufacturer's instructions.

### 2.9. DNA Extraction and Nested PCR Amplification of Src Exons 9–12

DNA was extracted from the parent MDA-MB-231 and the resistant 231DasB cell lines using the QIAamp<sup>®</sup> DNA mini kit (Qiagen, Hilden, Germany). Exons 9–12 of the *Src* gene were amplified using the primer sets in Table S1. The forward outer primer and the reverse primer were used in the first PCR reaction and the forward inner primer and the reverse primer were used in the second PCR reaction. The following PCR conditions were used for the first reaction: 5  $\mu$ L 10 $\times$  Amplitaq Gold Buffer

(Thermo-Fischer, Dublin, Ireland), 3  $\mu$ L 25 mM MgCl<sub>2</sub>, 1  $\mu$ L 10 mM dNTPs, 5  $\mu$ M each of forward and reverse primer, 0.25  $\mu$ L Amplitaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and 50 ng of DNA made up to a volume of 50  $\mu$ L with dH<sub>2</sub>O. A pre-PCR heat step of 95 °C for 5 min was carried out to activate the enzyme and the DNA was amplified for 35 cycles at 95 °C (1 min), 56 °C (1 min) and 72 °C (1 min) and at 72 °C (10 min) after the last cycle. The second PCR was carried out as above with 1  $\mu$ L of the first PCR reaction replacing the DNA. Ten microliters of the PCR product were electrophoresed on 1% agarose gel to verify product integrity. PCR products were purified using a QIAquick PCR purification kit (Qiagen). The DNA concentration was measured using the Nanodrop 1000 spectrophotometer (Thermo Fischer, Dublin, Ireland).

### 2.10. Cycle Sequencing of PCR Products

Cycle sequencing reactions were set up as follows: 2  $\mu$ L of BigDye<sup>®</sup> Terminator Mix v3.1, 20 ng amplicon DNA, 3.2 pmol of forward or reverse primer, 2  $\mu$ L sequencing buffer and diluted to 20  $\mu$ L with water. A positive control was also set up to ensure the efficiency of the sequencing reaction (1  $\mu$ L pGem, 2  $\mu$ L M13 primer, 2  $\mu$ L of BigDye<sup>®</sup> Terminator Mix v3.1 and 2  $\mu$ L sequencing buffer). The pGem and BigDye<sup>®</sup> Terminator v3.1 mix were both sourced from Applied Biosystems (Warrington, UK). Initial denaturation was carried out by a rapid thermal ramp to 96 °C (1 min), followed by 25 cycles of: rapid thermal ramp to 96 °C (10 s), rapid thermal ramp to 50 °C (5 s), rapid thermal ramp to 60 °C (4 min). Unincorporated dye terminators were removed before performing capillary electrophoresis using the DyeEx 2.0 Spinkit (Qiagen). Sequencing was performed on a 3130xl genetic analyser (Thermo-Fischer, Ireland, and sequencing files were analysed using the BioEdit v 7.0.8 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA).

### 2.11. Statistical Analysis

Alterations in doubling times of cell lines, changes in the invasive and migratory potential of cell lines, and alterations in proteomic signalling were measured using the Student's *t*-test. Error bars represent the standard deviation of triplicate experiments, where <sup>\*</sup>/<sup>\*\*</sup>/<sup>\*\*\*</sup> indicates a *p*-value of  $\leq 0.05/0.01$ , respectively, where a *p*-value  $< 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Dasatinib Exposure Induces a Resistant Phenotype

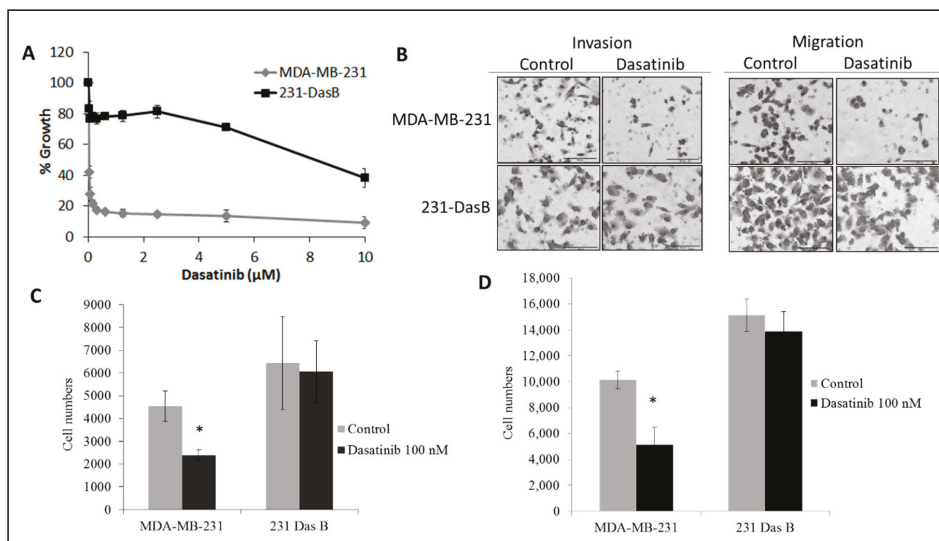
MDA-MB-231 cells are very sensitive to growth inhibition by dasatinib (IC<sub>50</sub> = 40  $\pm$  1 nM) [3]. A cell line model of acquired resistance to dasatinib, MDA-MB-231 cells, was developed by continuous exposure to dasatinib for approximately three months. Following this treatment the IC<sub>50</sub> value for dasatinib increased to greater than 5  $\mu$ M, confirming acquired resistance to dasatinib (IC<sub>50</sub> > 1  $\mu$ M) (8) compared to MDA-MB-231 cells (Figure 1A). The MDA-MB-231 cells that developed acquired resistance to dasatinib were referred to as 231 DasB.

No significant difference in growth rate was observed between the MDA-MB-231 parental cells and the 231-DasB cells. MDA-MB-231 cells show a significant dose dependent increase in doubling time in the presence of dasatinib, whereas the 231-DasB cells show no significant change in doubling time in response to dasatinib (Table 1).

**Table 1.** Doubling time in hours ( $\pm$  standard deviation) of MDA-MB-231 and MDA-MB-231-Das cells with and without dasatinib treatment. \* indicates *p* < 0.05 calculated using the Student's *t*-test.

Cell Line	Control	D 50 nM	D 100 nM
MDA-MB-231	17.6 $\pm$ 1.2	32.2 $\pm$ 3.3*	46.8 $\pm$ 5.1*
231 DasB	19.1 $\pm$ 2.4	21.0 $\pm$ 0.2	21.2 $\pm$ 3.1

We have previously shown that dasatinib treatment significantly decreases migration and invasion of MDA-MB-231 cells [3]. Migration, but not invasion, was significantly increased in the 231-DasB variant compared to the parental cell line ( $p = 0.009$ ) (Figure 1B). In the 231-DasB-resistant variant, dasatinib did not inhibit invasion ( $p = 0.772$ ) (Figure 1C) or migration ( $p = 0.340$ ) (Figure 1D).



**Figure 1.** Characterisation of the 231DasB cell line: (A) Proliferation assays of MDA-MB-231 and 231-DasB with serially decreasing concentrations of dasatinib from 10 µM; (B) representative images of MDA-MB-231 and 231-DasB post-18 h 100 nM dasatinib treatment in invasion and migration assays; (C) invasion assays and (D) migration assays of MDA-MB-231 and 231-DasB post-18 h 100 nM dasatinib treatment. Error bars represent the standard deviation of triplicate experiments. \*  $p \leq 0.05$ .  $p$  values were calculated using the Student's  $t$ -test.

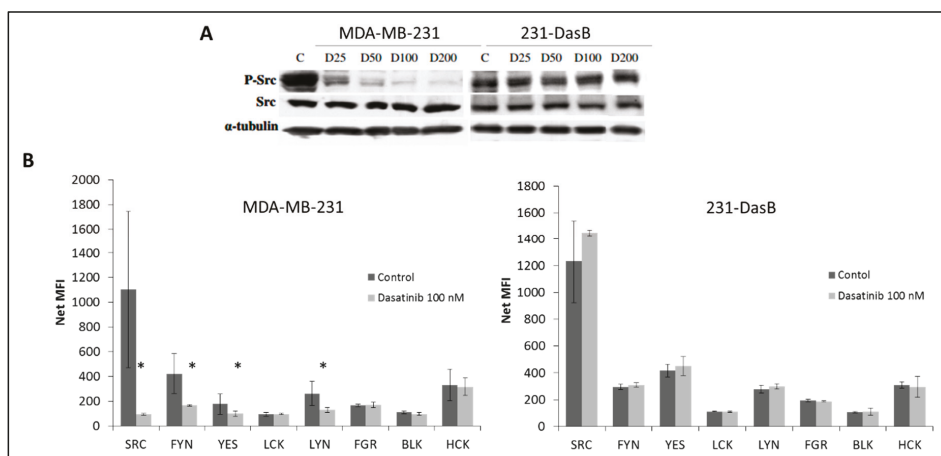
Dasatinib is a substrate for the drug efflux pumps BCRP and MDR-1 [15]. Therefore, in order to determine if drug efflux pumps were involved in the resistance to dasatinib in 231-DasB cells, we assessed the uptake of dasatinib in the parental MDA-MB-231 cells and the resistant variant, 231-DasB. Initially, dasatinib accumulation was measured using 2 µM dasatinib across a range of cell seeding numbers. While the relative mass of drug measured increased with decreasing cell density, no significant difference in accumulation between parental and variant cells was observed, suggesting drug pumps do not influence resistance to dasatinib in the 231-DasB cell line (Figure S1). To confirm this, we tested growth inhibition with dasatinib combined with the potent BCRP and MDR-1 inhibitor, elacridar [16]. Proliferation was measured in the parental and variant cell lines treated with dasatinib alone or in combination with a concentration of elacridar sufficient to inhibit BCRP [17] and MDR-1 [18]. The addition of elacridar did not cause a significant increase in dasatinib-induced growth inhibition in either the parental or the variant cell line (Figure S2).

### 3.2. Phosphorylation of Src Is Altered in Dasatinib-Resistant Cells

The 231-DasB cells were tested for cross-resistance to the Src kinase inhibitor PD180970. The 231-DasB cells showed a 2.2-fold increase in the PD180970  $IC_{50}$  compared to the MDA-MB-231 cells ( $876.1 \pm 74.4$  versus  $400.2 \pm 35.6$  nM,  $p = 0.003$ ), implicating Src in the resistant phenotype. Therefore, we examined phosphorylation of Src in the resistant cells. As expected, dasatinib treatment reduced the levels of p-Src (Y419) in MDA-MB-231 cells but no reduction in p-Src (Y419) was observed in the 231-DasB cells following dasatinib treatment (25–200 nM) (Figure 2A). The Src family kinases

(SFKs) consist of nine family members with high conservation between family members at tyrosine 419 (Y419). We examined the phosphorylation of seven of these proteins using magnetic multiplex assays. Consistent with the Western blot results, MDA-MB-231 showed a significant reduction in p-Src in response to dasatinib treatment (Figure 2B). p-FYN, p-YES and p-LYN were also significantly reduced after treatment with dasatinib. In the 231-DasB cells, treatment with dasatinib did not decrease the level of any of the phospho-SFK proteins examined (Figure 2B).

To determine if a mutation in the active site of Src kinase may be causing constitutive activation of p-Src, in the presence of dasatinib, we sequenced exons 9–12 of the *Src* gene, which encompass tyrosine 419 and the regulatory site at tyrosine 530 [19,20], in both the MDA-MB-231 parental and 231DasB cells. No alteration in the *Src* sequence was detected in the 231DasB cells (Figure S3).



**Figure 2.** (A) Levels of total Src and phosphorylated Src [pY419] in MDA-MB-231 (parental and 231-DasB cells treated with dasatinib for 6 h. (P-: phospho-; C: control (untreated); D: dasatinib (nM)). (B) Phosphorylation of Src family kinases as determined by multiplex bead assay in MDA-MB-231 and 231-DasB cell lines with and without 6-h dasatinib treatment (100 nM). NET MFI is net median fluorescence intensity. Error bars represent the standard deviations of triplicate independent experiments. \* indicates  $p < 0.05$  calculated using the Student's *t*-test.

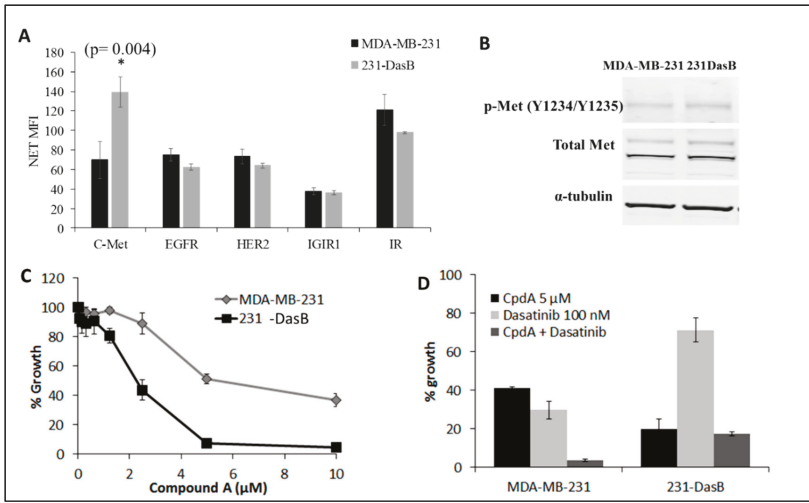
### 3.3. *cMet* Signalling Is Increased in Dasatinib-Resistant Cells

To investigate the possible role of upstream receptor tyrosine kinases (RTKs) in activation of Src kinase in the 231DasB cells, we examined the phosphorylation of a panel of RTKs using a multiplex assay. c-Met (panTYR) was the only RTK of the five tested that was altered in the 231-DasB cells. 231-DasB cells show significantly higher levels of p-Met ( $p = 0.004$ ) as determined by ELISA, compared to the MDA-MB-231 cells (Figure 3A). We then demonstrated a non-significant 1.4-fold increase ( $p = 0.08$ ) in phosphorylation of c-Met at its activation site (Y1234/Y1235) (Figure 3B). The difference in results between ELISA and Western blotting is likely due to the ELISA assay detecting all changes in p-Met activation, whilst we only analysed changes in p-Met (Y1234/1235) using Western blotting.

Based on the increased levels of p-Met in the 231-DasB cells, we examined sensitivity to a c-Met inhibitor, compound A (CpdA). The 231-DasB cells are more sensitive to CpdA, with an  $IC_{50}$  of  $1.4 \pm 0.5 \mu\text{M}$ , compared to the MDA-MB-231 cells where the CpdA  $IC_{50}$  is  $6.8 \pm 0.2 \mu\text{M}$  (Figure 3C).

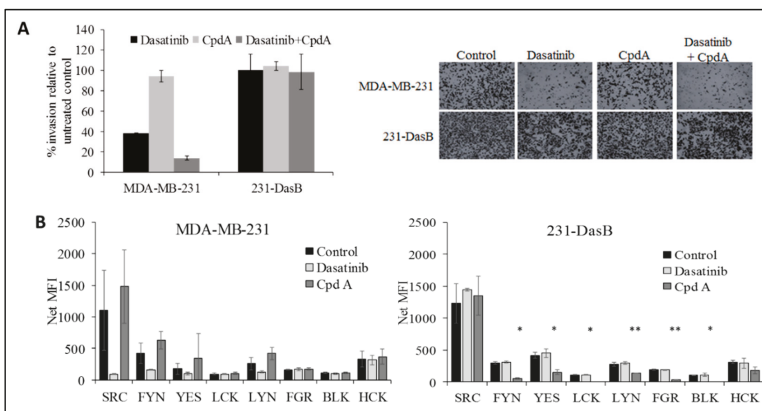
Combined treatment with CpdA and dasatinib resulted in significantly decreased growth in MDA-MB-231 ( $p = 0.02$ ) (Figure 3D). In the 231-DasB cells, the combination did not enhance growth inhibition compared to CpdA alone (Figure 3D).





**Figure 3.** (A) Phosphorylation of five RTKs in the MDA-MB-231 and 231-DasB cell lines, where NET MFI is net median fluorescence. Error bars represent the standard deviations of triplicate independent experiments. \* indicates  $p < 0.05$ .  $p$  values were calculated using the Student’s  $t$ -test. (B) Immunoblots for p-Met (Y1234/Y1235) and total Met in MDA-MB-231 and 231-DasB cells.  $\alpha$ -tubulin was used a loading control. (C) MDA-MB-231 and 231-DasB dose-response curves with serially decreasing concentrations of CpdA from 10  $\mu$ M; (D) Fixed concentration proliferation assays with 100 nM dasatinib and 5  $\mu$ M CpdA in MDA-MB-231 and 231-DasB cells.

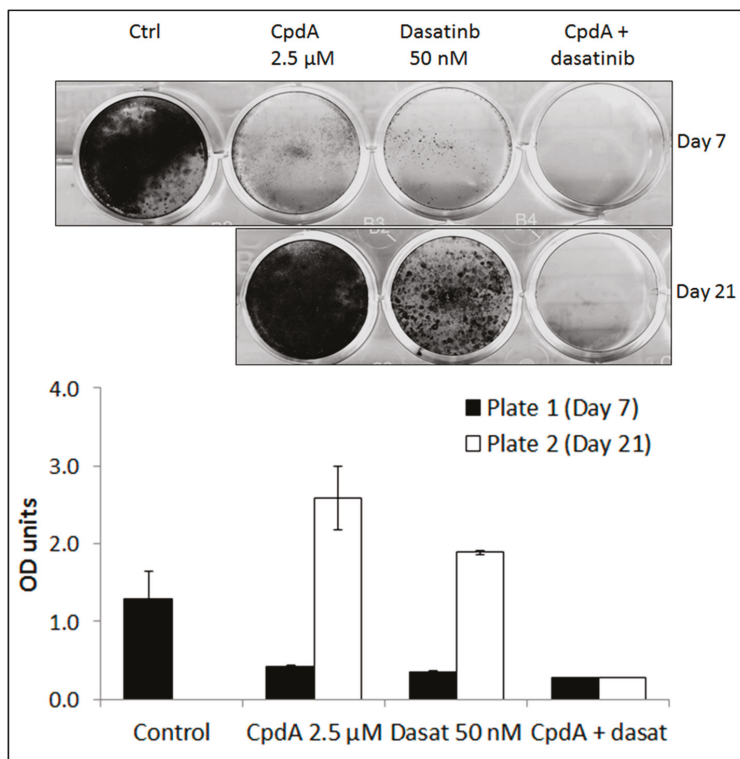
The combined treatment also significantly reduced invasion in the MDA-MB-231 cells ( $p = 0.002$ ), but not in the 231-DasB cells (Figure 4A). We examined the effects of CpdA on the phosphorylation of the SFKs. No significant change was observed in the parental MDA-MB-231 cells following treatment with CpdA; however, in 231-DasB cells six of the eight p-SFKs show a significant reduction in phosphorylation. FYN, YES, LCK, LYN, FGR and BLK all show significant reductions in phosphorylation in response to CpdA treatment (5  $\mu$ M) (Figure 4B).



**Figure 4.** (A) Invasion assays in MDA-MB-231 and 231-DasB cells with/without dasatinib (100 nM) and/or CpdA (5  $\mu$ M); (B) phosphorylation of SFKs in MDA-MB-231 and 231-DasB in response to dasatinib 100 nM and CpdA (5  $\mu$ M) where NET MFI is net median fluorescence intensity. \*  $p < 0.05$ , \*\*  $p < 0.01$ . Error bars represent the standard deviations of triplicate independent experiments.  $p$  values were calculated using the Student’s  $t$ -test.

### 3.4. cMET Inhibition Blocks Dasatinib Resistance

To determine if cMET inhibition may prevent the emergence of resistance to dasatinib in TNBC cells, the MDA-MB-231 cells were treated with CpdA alone (2.5  $\mu$ M), dasatinib alone (50 nM) or dasatinib plus CpdA. After seven days of treatment, both CpdA and dasatinib as single agents showed significant inhibition of growth (Figure 5). However, by day 21 cells treated with either CpdA or dasatinib started to grow again, suggesting the emergence of resistance, whereas the cells treated with CpdA combined with dasatinib showed no evidence of significant regrowth (Figure 5).



**Figure 5.** Short-term resistance assay in MDA-MB-231 cells treated with 2.5  $\mu$ M CpdA and/or 50 nM dasatinib for seven days and 21 days. Optical density (OD) was determined by measuring the absorbance of the crystal violet eluted from stained cells, at 590 nm. Error bars represent the standard deviation of triplicate experiments.

## 4. Discussion

Pre-clinical models of TNBC demonstrated significant sensitivity to the multi-targeted Src kinase inhibitor dasatinib (with specificity for BCR/Abl and Src family kinases); however, clinical trials with single-agent dasatinib showed limited efficacy in unselected populations [3,8,12]. In this study we sought to study the effects of long-term exposure to dasatinib on MDA-MB-231 cells, which are highly sensitive to dasatinib in three- or five-day proliferation assays [3,8]. Clinical studies indicated that significant resistance emerged within three months of continuous exposure to physiologically relevant concentrations of dasatinib [21,22]. Interestingly, we also demonstrated that resistance to dasatinib developed very quickly in the MDA-MB-231 cells. In HER2 positive breast cancer cell lines, we have found that resistance to HER2 targeted therapies generally emerges after approximately six months of

continuous exposure [23,24]. This rapid development of dasatinib resistance may contribute to the lack of clinical activity observed in the single-agent phase II clinical trials.

As dasatinib is a substrate for the drug efflux pumps BCRP and MDR-1 [15], we performed accumulation assays to determine if the concentration of dasatinib achieved in the resistant cells was lower than in the parental cells. No difference in dasatinib accumulation was observed in the resistant cells and inhibition of MDR-1 and BCRP by elacridar did not enhance response to dasatinib in the resistant cells. Taken together, these results suggest that neither altered drug uptake nor efflux plays a role in the acquired dasatinib-resistant phenotype in these cells.

In the 231-DasB cells phosphorylation of Src at Y419 is not inhibited by dasatinib. This site is highly conserved across the SFKs. Therefore, to examine if specific SFKs are altered in the resistant cells we performed a multiplex assay for seven SFK members. Src was the only member of the SFK family that showed increased phosphorylation in the resistant cells. While dasatinib inhibited phosphorylation of several of the SFKs in MDA-MB-231 cells, it did not inhibit phosphorylation of any of the SFKs in the resistant cells.

Activation of Src kinase has been documented in a number of cancer types; however, activating mutations are rare. A truncating mutation in Src at Y530 has been identified in small subsets of colon and endometrial cancers [20,25]. To determine if a mutation in Src could cause constitutive Src phosphorylation in 231-DasB cells, we sequenced exons 9–12 of the *Src* gene, which encompass the kinase domain and the regulatory site at tyrosine 530 [19]. No mutations were detected in the *Src* gene in the 231-DasB cell line.

Altered receptor tyrosine kinase signalling, in particular EGFR and c-Met signalling have been implicated in increased pSrc signalling in cancer, particularly in lung cancer but also in breast cancer [26,27]. Therefore, we examined whether altered RTK signalling might play a role in the dasatinib-resistant phenotype. Of the five RTKs examined, only c-MET showed an increase in phosphorylation in the resistant cells. Although EGFR has been implicated in cross-talk with both Src and MET, pEGFR levels were unaltered in the 231-DasB cells suggesting that EGFR is not a mediator of the dasatinib-resistant phenotype.

Recently another TNBC model of dasatinib resistance has been described, using the BT20 cell line. Pinedo-Carpio et al. found that chronic exposure to dasatinib resulted in increased expression of TGF $\beta$ 2 and increased resistance to a TGF $\beta$  inhibitor, with a shift towards non-canonical TGF $\beta$  signalling [28]. Zhang et al. have previously shown that non-canonical TGF $\beta$  signalling can stimulate phosphorylation of Src at Tyr419 in a lung cancer cell line [29]. Thus, it is possible that non-canonical TGF $\beta$  signalling may also play a role in the dasatinib resistance in our 231-DasB model. The MDA-MB-231 cell line used in our analysis represents a post-EMT cell line [1], whilst the BT20 dasatinib-resistant model [2] is not an epithelial-mesenchymal transition (EMT) cell line. Finn et al. reported that the majority of post-EMT TNBC cell lines are highly sensitive to dasatinib, similar to MDA-MB-231. Pinedo-Carpio et al. [2] did not report any changes in cMET expression as a result of acquired dasatinib resistance, indicating that the changes we observed in cMet in the 231 DasB cells may be unique to post-EMT cells in response to dasatinib treatment. Consistent, however, with the increased p-Met levels, the 231-DasB cells also showed increased sensitivity to the Met inhibitor Cpda. Interestingly, the combination of Cpda and dasatinib resulted in significantly enhanced growth inhibition in the parental cell line but not in the 231-DasB cells. Furthermore, short-term resistance assays in the MDA-MB-231 cells showed that, in the presence of compound A, dasatinib resistance did not emerge, suggesting that c-Met inhibition may sensitise the cells to dasatinib and prevent the delay or block the development of dasatinib resistance. Cpda also enhanced dasatinib-mediated inhibition of invasion in the parental cells but not in the resistant cells. In 231-DasB cells, Cpda inhibits phosphorylation of several members of the SFK family—FYN, YES, LCK, LYN, FGR and BLK—but did not inhibit phosphorylation of Src. Our results suggest that pSrc signalling may be critical in the development of the aggressive phenotype of TNBC, and that the inhibition of p-Src may be required to overcome established resistance to dasatinib and possibly the aggressive, invasive behaviour of TNBC.

c-Met has previously been implicated in other models of acquired resistance to other small molecule inhibitors, chemotherapy and radiotherapies. Cabozantinib, a small-molecule c-Met inhibitor, has been shown to overcome gemcitabine resistance in pancreatic cancer and, quite encouragingly, displayed only a very low level of acquired resistance despite long-term treatment [30]. A similar situation was observed in two primary multiple myeloma cell lines that show increased p-Met at the development of resistance compared to the sensitive cell lines [31]. In the MDA-MB-231 cell line, ionising radiation increases the activation of c-Met and targeting the cells with a c-Met inhibitor sensitises the cells to radiation therapy [32]. Met has also been implicated in the resistance to the antiangiogenic therapy bevacizumab in glioblastomas [33]. Upregulation of c-Met was noted after exposure to bevacizumab. Our results suggest that, while c-Met may be a potential target in the acquired resistance setting, it may also be appropriate to target c-Met in combination with other therapies to prevent the development of resistance.

Dasatinib has been previously tested and has failed clinical trials in breast cancer; however, combining dasatinib with a c-Met inhibitor may be a rational therapeutic strategy to test in TNBC. Further evaluation in preclinical models of TNBC, including in vivo, would be required to support the progression of this combination into clinical trials. Furthermore, the evaluation of changes in the phosphorylation of c-Met following dasatinib treatment in tumours from patients enrolled on previous dasatinib clinical trials would be important. These trials, which included sample collection for biomarker studies ([11], NCT00817531), would elucidate whether the increase in p-Met levels that we observed in vitro following dasatinib treatment also occurs in TNBC cells in patients following dasatinib exposure. If increases in p-Met were detected in dasatinib-treated tumours, it would support clinical testing of dasatinib plus a c-Met inhibitor to block the development of dasatinib resistance.

## 5. Conclusions

In conclusion, our results suggest that cMet may be a rational target in triple-negative tumours that have developed acquired resistance to dasatinib; or, perhaps a better approach may be to combine dasatinib and cMet inhibition before metastasis occurs to improve the response to dasatinib and potentially block the emergence of resistant cells.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/11/4/548/s1>, Figure S1: Dasatinib accumulation assays in MDA-MB-231 parental and resistant variant cells. Cells seeded at different densities were treated with 2  $\mu$ M dasatinib for two hours. Cells were then counted using the Guava Easycyte flow cytometry. The mass of dasatinib per sample was measured by mass spectrometry and expressed as ng per million cells. Error bars represent standard deviations of three replicate flasks for each seeding density, Figure S2: Proliferation assays in MDA-MB-231 parental (A) and 231-DasB variant (B) cells treated with dasatinib (Das) alone and combined with a fixed concentration of elacridar (Elac). Error bars represent the standard deviations of triplicate experiments, Figure S3: *Src* exon 9 sequence trace, Table S1: Primer Sequences for Nested PCRs.

**Author Contributions:** Conceptualization: N.O., J.C., M.J.D.; Methodology: P.G., N.M., B.C., A.J.E., K.G., S.R., N.O.; Formal analysis: P.G., N.M., B.C., A.J.E., K.G., S.R., N.O.; Investigation: P.G., N.M., B.C., A.J.E., K.G., S.R.; Data curation: P.G., N.M., B.C., A.J.E., K.G., S.R., N.O.; Writing—original draft preparation: P.G., N.M., B.C., S.R.; Writing—review and editing: K.G., R.O., K.J.O., N.W., M.J.D., J.C., N.O.; Supervision: R.O., K.J.O., N.W., M.J.D., J.C., N.O.; Funding acquisition: N.O., J.C.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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Article

# Should All Patients With HR-Positive HER2-Negative Metastatic Breast Cancer Receive CDK 4/6 Inhibitor As First-Line Based Therapy? A Network Meta-Analysis of Data from the PALOMA 2, MONALEESA 2, MONALEESA 7, MONARCH 3, FALCON, SWOG and FACT Trials

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**Abstract:** *Background:* We aim to understand whether all patients with hormonal receptor (HR)-positive (+)/human epidermal growth factor receptor-2 (HER2)-negative (−) metastatic breast cancer (MBC) should receive cyclin D-dependent kinase (CDK) 4/6 inhibitor-based therapy as a first-line approach. *Methods:* A network meta-analysis (NMA) using the Bayesian hierarchical arm-based model, which provides the estimates for various effect sizes, were computed. *Results:* First-line treatment options in HR+/HER2− MBC, including CDK 4/6 inhibitors combined with aromatase inhibitors (AIs) or fulvestrant (F), showed a significantly longer progression-free survival (PFS) in comparison with AI monotherapy, with a total of 26% progression risk reduction. In the indirect comparison across the three classes of CDK 4/6 inhibitors and F endocrine-based therapies, the first strategy resulted in longer PFS, regardless of specific CDK 4/6 inhibitor (HR: 0.68; 95% CrI: 0.53–0.87 for palbociclib + AI, HR: 0.65; 95% CrI: 0.53–0.79 for ribociclib + AI, HR: 0.63; 95% CrI: 0.47–0.86 for abemaciclib + AI) and patient's characteristics. Longer PFS was also found in patients with bone-only and soft tissues limited disease treated with CDK 4/6 inhibitors. *Conclusions:* CDK 4/6 inhibitors have similar efficacy when associated with an AI in the first-line treatment of HR+ MBC, and are superior to either F or AI monotherapy, regardless of any other patients or tumor characteristics.



**Keywords:** palbociclib; ribociclib; abemaciclib; fulvestrant; aromatase inhibitors; metastatic breast cancer

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## 1. Introduction

Hormone receptor (HR)-positive (+) and human epidermal growth factor receptor type (HER2)-negative (−) metastatic breast cancer (MBC) represents the most common invasive cancer subtype in women [1]. Almost two-thirds of women with newly diagnosed MBC have HR+ tumors, and approximately 25% of women with an early HR+ breast cancer diagnosis eventually relapse after adjuvant treatments [2].

As the role of estrogens in etiology and breast cancer progression is well-established, the modification of estrogen activity has represented the treatment of choice in women with HR+ MBC for several years, particularly for those with slowly progressive disease and limited tumor-related symptoms [3]. Selective estrogen receptor modulators (such as tamoxifen) [4], selective estrogen receptor down-regulators (SERDs, like fulvestrant [F]) [5,6] aromatase inhibitors (AIs; such as letrozole [7], anastrozole [8] or exemestane [9]) are the mainstay of anticancer endocrine therapy (ET) [10]. The recent addition of CDK 4/6 inhibitors to standard ET has further improved outcomes in both first- and later-line therapy settings. CDK 4/6 inhibitors act by inactivating the complex CDK-D-type cyclins (CCND), leading to an increase in the retinoblastoma protein (pRb), which negatively regulates E2F transcriptional factors, eventually resulting in the inhibition of cell cycle progression and apoptosis of tumor cells [11].

In the first-line setting, CDK 4/6 inhibitors, which mechanistically work in different ways through estrogen receptor interference, have been studied in combination with AIs in the PALOMA-2 [12], MONALEESA-2 [13], and MONARCH-3 studies in the context of postmenopausal women [14], as well as in premenopausal women, in combination with either tamoxifen or an AI in the MONALEESA-7 study [15]. In particular, palbociclib, ribociclib, and abemaciclib, the three available CDK 4/6 inhibitors, in combination with standard ET, showed progression-free survival (PFS) improvement in phase III trials, and have been approved for use in first and in later lines of therapy in women with HR+HER2−MBC, regardless of menopausal status, age, endocrine sensitivity and type of metastasis [12,16]. Interestingly, although the mechanism of action of these CDK 4/6 inhibitors is similar, they also present some differences. In fact, while palbociclib and ribociclib have a similar chemical structure, abemaciclib is 14-times more potent against CDK 4 compared with CDK 6 and presents a higher selectivity for the complex CDK 4/cyclin D1 [11].

Despite the progress made in efficacy, combination therapy also resulted in increased toxicities, costs, and tighter clinical monitoring for patients [17]. In addition, the improvement in PFS has not yet translated into an increase in overall survival (OS) for all studies focusing on first-line settings [18–20].

Therefore, how and when to incorporate CDK 4/6 inhibitors in the complex management of HR+HER2−MBC remains one of the main unmet clinical need in this setting [21].

Indeed, single-agent ET yielded a median PFS ranging from 14 to 16 months in the control arms of the first-line trials with CDK 4/6 inhibitors. Thus, the fact that for some patients, the addition of CDK 4/6 inhibitors might be avoided is a debated clinical topic. Moreover, according to the Fulvestrant and Anastrozole Compared in Hormonal Therapy Naive Advanced Breast Cancer (FALCON) trial, comparing F with anastrozole in the same setting, the single-agent F therapy may be a further reasonable option for HR+/HER2−MBC patients who are ET-naïve, especially those with the non-visceral disease. Indeed, the median PFS of 16.6 months reported in the F arm compared with 13.3 months in the anastrozole arm, which was observed in the context of the general population, was even higher in patients without visceral disease (22.3 versus 13.6 months; hazard ratio [HR]: 0.59; 95% CI: 0.42–0.84) [5].

The ongoing studies, which are evaluating predictive markers of endocrine resistance or sensitivity, will probably provide enough evidence for helping in the clinical decision-making and for a better definition of the optimal ET-based strategy for this specific set of luminal breast cancer [22–24].

In the meantime, in our current daily clinical practice, also considering minor or uncertain differences in efficacy between the available CDK 4/6 inhibitors, the choice of first-line ET strategy is essentially based on one of the three CDK 4/6 inhibitors according to their specific toxicity profile and patient comorbidities or preferences.

Considering the lack of formal and reliable comparisons between the three CDK 4/6 inhibitors, in addition to the lower toxicity profile of F, the aim of this systematic review and meta-analysis is the indirect comparison between the combination strategy, including CDK 4/6 inhibitors plus AI [12–15], and F-based therapies for the first-line treatment of HR+/HER2– MBC [5,25–27].

## 2. Materials and Methods

### 2.1. Search Strategy and Study Selection

We followed the PRISMA statement for reporting systematic reviews and meta-analysis. Two authors independently examined the abstracts retrieved by a search strategy in electronic databases (MEDLINE, EMBASE, and The Cochrane Central Register of Controlled Trials) from November 2011 to June 2019. We used the following search string ((metastatic breast cancer) AND (CDK 4/6 inhibitor OR endocrine therapy OR aromatase inhibitor OR letrozole OR anastrozole OR exemestane OR tamoxifen OR F OR palbociclib OR everolimus OR ribociclib OR abemaciclib). The research was conducted on 5 June 2019. Proceedings of the American Society of Clinical Oncology (ASCO) Annual Meeting, San Antonio Breast Cancer Annual Symposium, and the European Society of Medical Oncology Annual Meeting were also queried from November 2011 to June 2019 for relevant abstracts. In cases where a report of the same trial was obtained, the most recent results were included (corresponding to longer follow-up). Then, the authors examined full-text articles of potentially eligible studies according to the eligibility criteria. Disagreements on the inclusion of selected trials were resolved in discussions with another author. This article does not contain any studies with human participants or animals performed by any of the authors. It was unnecessary, given the study does not contain any studies with human participants or animals performed by any of the authors.

### 2.2. Eligibility Criteria

We decided only to include phase III randomized controlled trials (RCTs) that reported the comparison of CDK 4/6 inhibitors plus ET or F plus or less ET versus ET treatment alone as first-line treatment in HR+/HER2– MBC. We also excluded trials with incomplete data or different control arm.

### 2.3. Outcomes

The primary outcome was PFS, calculated from the date of randomization to the date of progression (defined by the Response Evaluation Criteria in Solid Tumors “RECIST” 1.1 criteria or death). The secondary outcomes were: (1) objective response rate (ORR): defined as the percentage of patients with complete or partial response as per RECIST 1.1 criteria (as assessed in all randomly assigned patients); (2) clinical benefit (CB): defined as a confirmed complete or partial response or stable disease lasting 24 weeks or more; (3) OS: defined as the time from randomization to death from any causes. Subgroup meta-regression analysis was also conducted for PFS indirect comparison according to age, Eastern Cooperative Oncology Group (ECOG) performance status, ethnicity, prior chemotherapy or ET exposure, measurable disease at the time of metastasis occurrence, visceral or bone-only disease, time from the initial diagnosis of breast cancer to metastasis onset.

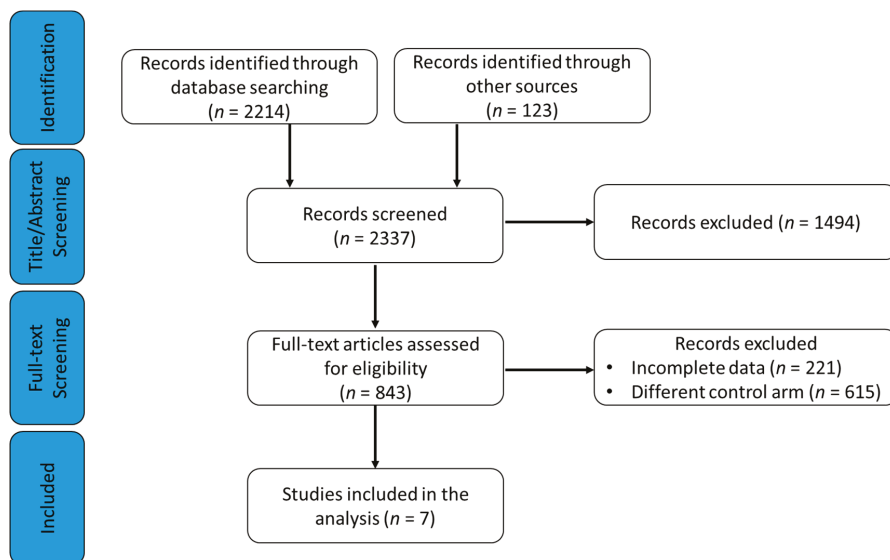
## 2.4. Data Collection and Statistical Analysis

A network meta-analysis (NMA) was carried out utilizing the method in the study by Valkenhof et al. [28], which performed NMA using the Bayesian hierarchical arm-based model and provides estimates for various effect sizes. For PFS, HR and 95% credible interval (CrI) were reported. In addition, ORR and CB rates were reported, and the results were expressed as odds ratios (OR) with their 95% CrI. The NMA plot, in which treatments directly compared were connected with straight line was generated. NMAs on patients' subgroups were also performed. All analyses were performed using R Statistical Software version 3.4.3 along with the gemtc package, which uses Markov chain Monte Carlo (MCMC) techniques through Just Another Gibbs Sampler (JAGS) [29].

## 3. Results

### 3.1. Study Selection

Through the search strategy, we identified four phase III trials comparing 1441 patients treated with CDK 4/6 inhibitors (palbociclib, ribociclib, abemaciclib) in combination with an AI (1106 patients) [12–14], an AI plus ovarian function suppression (OFS; 248 premenopausal patients), or tamoxifen plus OFS (87 premenopausal patients) [15]. Three other phase III randomized controlled trials (RCTs) compared 837 patients treated with F alone (230 patients) or in combination with AI (607 patients) versus a total of 1891 patients treated with AI alone (letrozole 2.5 mg daily or anastrozole 1 mg per day on a continuous schedule), tamoxifen plus OFS (90 premenopausal patients) or AI plus OFS (247 premenopausal) [5,25,27]. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram for study inclusion is shown in Figure 1.

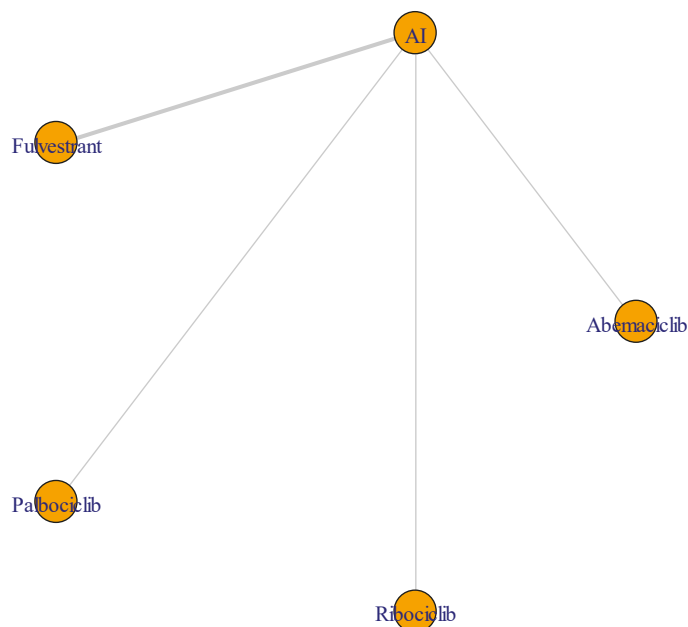


**Figure 1.** The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart summarizing the process for the identification of the eligible studies.

### 3.2. Description of Studies and Patients

Table 1 summarizes the main characteristics and outcomes of each trial. Palbociclib and ribociclib were tested in combination with letrozole 2.5 mg/day in PALOMA-2 and MONALEESA-2, respectively. Abemaciclib was used in combination with anastrozole 1 mg/day (19.9%) or letrozole 2.5 mg/day (79.1%) as per the physician's choice in the MONARCH-3 trial. Furthermore, ribociclib was also

studied in premenopausal patients in the context of the MONALEESA-7 trial. Specifically, in this study, ribociclib was combined with tamoxifen plus goserelin (26%) or with letrozole 2.5 mg/day or anastrozole 1 mg/day plus goserelin (74%). Eventually, F alone or in combination with anastrozole 1 mg/day was compared with the AI in the FALCON, Southwest Oncology Group (SWOG), and Fulvestrant and Anastrozole Combination Therapy (FACT) trials. The primary outcome was PFS in all trials (Table 1); secondary outcomes (ORR, CB rate, OS) in all trials were also reported in Table 1, while the NMA core design is shown in Figure 2. The network plot in Figure 2 offers a visual representation of the evidence. Nodes represent treatments, and edges represent the available direct comparisons—that is, they connect treatments that are directly compared in studies.



**Figure 2.** The network meta-analysis design: Network plot of the network meta-analysis. AI: Aromatase inhibitor.

Table 1. Main characteristics and outcomes of the seven eligible trials included for meta-analysis.

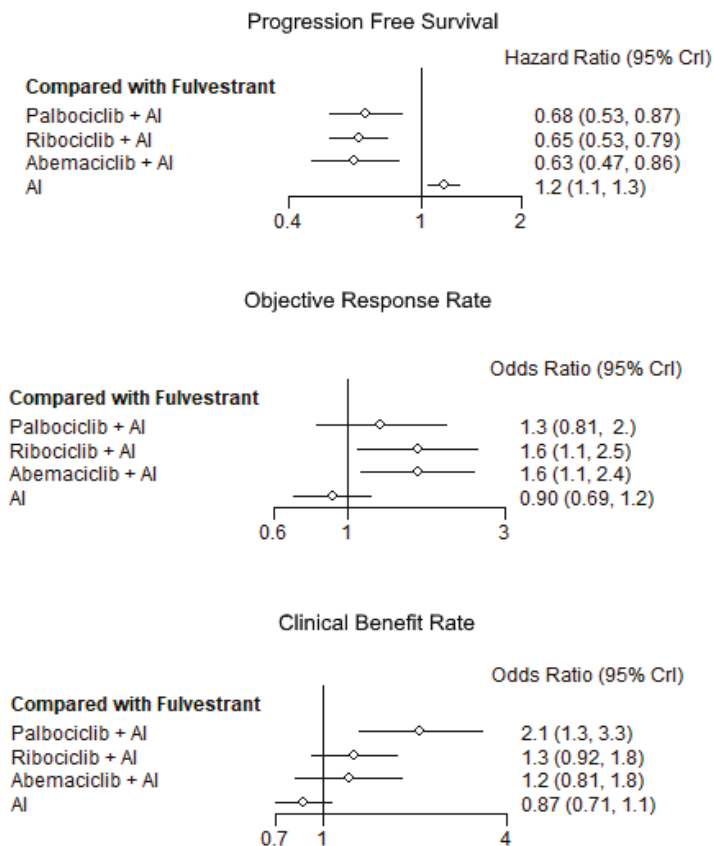
Study and First Author	Publication Year	Phase	Setting	Post-Menopausal	RR (%)	CB (%)	Median PFS (months)	Median OS (months)
PALOMA-2 Finn [12]	2016	III	First-line therapy for MBC in patients not treated before for their metastatic disease.	Yes	42.1 vs. 34.7	84.9 vs. 70.3	24.8 vs. 14.5	NR
MONALEESA-2 Hotobagyi [13]	2016 2018	III	First-line therapy for locally advanced and MBC. Patients who had not received previous systemic therapy for advanced disease were eligible. Previous neoadjuvant or adjuvant therapy with a nonsteroidal AI was not allowed unless the disease-free interval was more than 12 months	Yes	40.7 vs. 27.5 42.5 vs. 28.7	79.6 vs. 72.8 79.9 vs. 73.1	NR vs. 14.7 25.3 vs. 16	NR NR vs. 33
MONARCH-3 Goetz [14]	2017	III	First-line therapy for locally advanced and MBC (endocrine therapy in the neoadjuvant or adjuvant setting was permitted if the patient had a disease-free interval 12 months from the completion of endocrine therapy)	Yes	48.2 vs. 34.5	78 vs. 71.5	NR vs. 14.7	NR
MONALEESA-7 Tripathy [15]	2018 2019	III	First-line therapy for locally advanced or MBC (endocrine therapy and chemotherapy in the adjuvant or neoadjuvant setting was permitted, as was up to one line of chemotherapy for advanced disease).	Not (only pre and perimenopausal women were included and treated with goserelin)	41 vs. 30	79 vs. 70	23.8 vs. 13.0	NR NR vs. 40.9
FALCON Robertson [5]	2016	III	First-line therapy for locally advanced or MBC (no previous adjuvant therapy was admitted, only a first-line CHT for metastatic disease was accepted)	Yes	46 vs. 45	78 vs. 74	16.6 vs. 13.8	NR
SWOG Mehta [26]	2012 2019	III	First line for de novo MBC or recurrent MBC after 12 months by the end of adjuvant CHT or HT	Yes	27 vs. 22	73 vs. 70	15 vs. 13.5 15 vs. 13.5	47.7 vs. 41.3 49.8 vs. 42
FACT Bergh [27]	2012	III	First-line therapy in recurrent MBC after or during primary treatment (with or without HT, CHT, RT). Patients treated with an adjuvant AI had to be relapse-free for more than one year after completion of this type of endocrine therapy (30.2% of the patients in the experimental arm were endocrine naive)	Yes (only 3% of patients wer in the premenopausal status and were treated with GnRH agonists)	31.8 vs. 33.6	55 vs. 55.1	10.8 vs. 10.2	37.8 vs. 38.2

AI: aromatase inhibitor; CB: clinical benefit; CHT: chemotherapy; HT: hormonal therapy; MBC: metastatic breast cancer; GnRH, gonadotropin-releasing hormone; NR: not reported; OS: overall survival; PFS: progression-free survival; RR: response rate; RT: radiotherapy.

### 3.3. Outcomes

#### 3.3.1. Progression-Free Survival

As shown in the indirect comparison between CDK 4/6 inhibitors versus F-based therapies, the first strategy resulted in longer PFS, regardless of the specific CDK 4/6 inhibitor (HR: 0.68; 95% CrI: 0.53–0.87 for palbociclib + AI, HR: 0.65; 95% CrI: 0.53–0.79 for ribociclib + AI, HR: 0.63; 95% CrI: 0.47–0.86 for abemaciclib + AI) (Figure 3; top)



**Figure 3.** Forest plots with direct comparisons against fulvestrant for progression-free survival, objective response rate, and clinical benefit rate. AI: Aromatase inhibitor.

#### 3.3.2. Objective Response

The CDK 4/6 inhibitors combination strategies resulted in higher RR in indirect comparison with F (OR:1.3; 95% CrI: 0.81–2.0 from palbociclib + AI versus AI: OR:1.6; 95% CrI: 1.1–2.5 from ribociclib + AI versus AI: OR:1.6; 95% CrI: 1.1–2.4 from abemaciclib + AI versus AI). (Figure 3; middle)

#### 3.3.3. Clinical Benefit

The CDK 4/6 inhibitor combination strategies resulted in higher CB in indirect comparison with F (OR: 2.1; 95% CrI: 1.3–3.3 from palbociclib + AI versus AI: OR: 1.3; 95% CrI: 0.92–1.8 from ribociclib + AI versus AI: OR:1.2; 95% CrI: 0.81–1.8 from abemaciclib + AI versus AI). (Figure 3; bottom).

## 3.3.4. Overall Survival

For overall survival (OS), no indirect comparison by NMA was performed because not all the studies data were completely mature. Indeed, data on OS have been recently reported in the MONALEESE-7 study where the estimated OS at 42 months was 70.2% (95% CI 63.5–76.0) in the CDK 4/6 inhibitor arm versus 46% (95% CI: 32–58.9) in the ET alone arm [18,19]. In addition, also the SWOG trial has recently shown the median OS of 49.8 months in 71% of the patients receiving F combination strategy vs. 42 months in 76% of the patients receiving ET monotherapy alone [23].

## 3.3.5. Safety Profile

The main adverse effects registered in each trial are reported in Table 2.

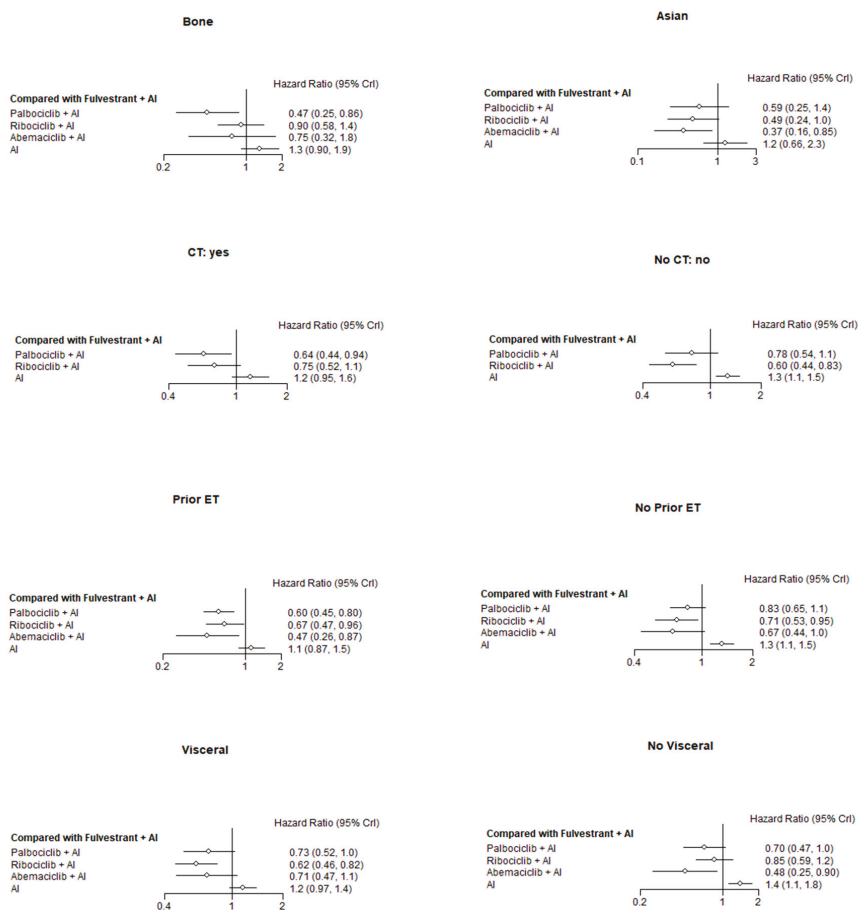
**Table 2.** Main adverse effects registered in each trial.

Study	Treatment	AEs	G3 (%)	G4 (%)
PALOMA-2	Palbociclib–letrozole	Any AEs	62.2%	13.5%
		Neutropenia	56.1%	10.4%
Leukopenia		24.1%	0.7%	
	Placebo–letrozole	Any AEs	22.1%	2.3%
		Neutropenia	0.9%	0.5%
MONALEESA-2	Ribociclib–letrozole	Neutropenia	52.4%	9.6%
		Abnormal LFT	8.4%	1.8%
		Leukopenia	20.1%	1.2%
	Placebo–letrozole	Abnormal LFT	2.4%	–
		Neutropenia, anemia, arthralgia	1.2%	–
MONARCH-3	Abemaciclib–nonsteroidal AI	Any AEs	51.7%	6.7%
		Neutropenia	22.0%	1.8%
		Leukopenia	8.3%	0.3%
		ALT increase	6.1%	0.3%
	Placebo–nonsteroidal AI	Any AEs	22.4%	2.5%
		Neutropenia	0.6%	0.6%
MONALEESA-7	Ribociclib group	Any AEs	63%	14%
		Neutropenia	51%	10%
		Leukopenia	13%	1%
	Placebo group	Any AEs	26%	4%
		Neutropenia	3%	1%
FALCON Robertson	Fulvestrant	Arthralgia (17%) Hot flush, fatigue, nausea (11%) Back pain (9%)		
	Anastrozole	Arthralgia, hot flush, nausea (10%)		
SWOG Mehta	Anastrozole	Musculoskeletal pain, fatigue, hot flashes, mood alterations, GI symptoms	15% (each 1–4%)	
	Anastrozole–fulvestrant	Musculoskeletal pain, fatigue, hot flashes, mood alterations, GI symptoms	13% (each 1–4%)	
FACT Bergh	Anastrozole	GI symptoms (25.2%) Joint disorders (27.6%) Hot flashes (13.8%)		
	Anastrozole–fulvestrant	GI symptoms (28.9%) Joint disorders (26.6%) Hot flashes (24.6%)		

ALT: alanine aminotransferase; AE: adverse event; AI: aromatase inhibitor; GI: gastrointestinal; G3: grade 3; G4: grade 4; LFT: liver function test.

### 3.4. Subgroup Analyses

Table 3 summarizes the main patient's characteristic according to subgroups analysis in each trial. Subgroup NMA among the three classes of CDK 4/6 inhibitors and F is reported in Figure 4.



**Figure 4.** Forest plot on treatment effect for progression-free survival by subgroups in the indirect comparison between CDK 4/6 inhibitors, aromatase inhibitors, and fulvestrant. AI: Aromatase inhibitor; CT: Chemotherapy; ET: Endocrine therapy.

For PFS analysis of the seven selected phase III RCTs [5,12–15,25,27] according to prespecified subgroups, a total of 2278 patients were in the CDK 4/6 inhibitors or fulvestrant arm and a total of 1891 patients were in ET arm alone. Among them, 335 patients in the CDK 4/6 inhibitors and 337 patients in ET arms alone were premenopausal. The indirect comparison between CDK 4/6 inhibitors combination strategies and F-based therapies showed quite consistent PFS improvements in favor of CDK 4/6 inhibitors in all subgroups. With reference to the most important NMA aim, compared with F-based therapy, the CDK 4/6 inhibitor combination strategy was associated with PFS improvement also in patients with disease limited to the bone or in non-visceral sites. Although no statistically significant difference emerged among the three classes of inhibitors in indirect comparison, NMA results also suggested a different potential tropism among them, which should be further investigated in prospective clinical trials.



Table 3. Main characteristics of the patients included in the randomized trials evaluated for the present network meta-analysis.

Characteristics	PALOMA-2, n (%)		MONALEESA-2, n (%)		MONARCH-3, n (%)		MONALEESA-7, n (%)		FALCON <sup>3</sup> , n (%)		SWOG b, n (%)		FACT4, n (%)	
	L. + Palb.	L.	L. + Rib.	L.	L or A. + Abem.	L. or A.	Rib. + T or nAls	F.	A.	F. + A.	A.	F. + A.	A.	A.
No. of patients	444	222	334	334	328	165	335	337	230	232	345	349	258	256
Age:														
• Median (range), years	62 (3089)	61 (28–88)	62 (23–91)	63 (29–88)	63 (58–87)	63 (32–88)	43 (25–58)	45 (29–58)	64 (38–87)	62 (36–90)	65 (36–91)	65 (27–92)	65 (33–86)	63 (36–90)
• <65 years	263 (59.2)	141 (63.5)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	124 (48.1)
• ≥65 years	181 (40.8)	81 (36.5)	NR	NR	NR	NR	NR	NR	108 (47)	91 (39)	NR	NR	NR	134 (51.9)
Ethnicity:														
• White	344 (77.5)	172 (77.5)	269 (80.5)	280 (83.8)	186 (56.7)	102 (61.8)	187 (56)	201 (60)	175 (76)	174 (75)	NR	NR	NR	242 (93.8)
• Asian	65 (14.6)	30 (13.5)	28 (8.4)	23 (6.9)	103 (31.4)	45 (27.3)	99 (30)	99 (29)	36 (16)	34 (15)	NR	NR	NR	4 (1.6)
• Black	8 (1.8)	3 (1.4)	10 (3.0)	7 (2.1)	NR	NR	10 (3)	9 (3)	NR	NR	NR	NR	NR	1 (0.4)
• Other	27 (6.1)	17 (7.7)	27 (8.1)	24 (7.2)	11 (3.4)	7 (4.2)	39 (12)	28 (8)	19 (8)	24 (10)	NR	NR	NR	11 (4.3)
Hormone receptor status:														
• ER+, PgR+	NR	NR	NR	NR	255 (77.7)	127 (77.0)	290 (87)	288 (85)	175 (76)	179 (77)	NR	NR	193 (74.8)	195 (76.2)
• ER+, PgR–	NR	NR	NR	NR	70 (21.3)	36 (21.8)	NR	NR	44 (19)	43 (19)	NR	NR	60 (23.3)	51 (19.9)
• Unknown	NR	NR	NR	NR	NR	NR	NR	NR	10(4)	7 (3)	NR	NR	4 (1.6)	6 (2.3)
• ER–PgR+	NR	NR	NR	NR	NR	NR	NR	NR	1 (<1)	3 (1)	NR	NR	1 (0.4)	4 (1.6)
Performance status:														
• ECOG 0	257 (57.9)	102 (45.9)	205 (61.4)	202 (60.5)	192 (58.5)	104 (63.0)	245 (73)	255 (76)	117 (51)	115 (50)	NR	NR	NR	NR
• ECOG 1	178 (40.1)	117 (52.7)	129 (38.6)	132 (39.5)	136 (41.5)	61 (37.0)	87 (26)	78 (23)	106 (46)	105 (45)	NR	NR	NR	NR
• ECOG 2	9 (2.0)	3 (1.4)	NR	NR	NR	NR	0	1 (<1)	7 (3)	12 (5)	NR	NR	NR	NR
• ECOG > 2	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
• Unavailable	NR	NR	NR	NR	NR	NR	3 (1)	3 (1)	NR	NR	NR	NR	NR	NR

Table 3. *Contd.*

Characteristics	PALOMA-2, n (%)		MONALEESA-2, n (%)		MONARCH-3, n (%)		MONALEESA-7, n (%)		FALCON <sup>3</sup> , n (%)		SWOG <sup>b</sup> , n (%)		FACT4, n (%)	
	L. + Palb.	L.	L. + Rib.	L.	L. or A. + Abem.	L. or A.	Rib. + T or nAls	F.	A.	F. + A.	A.	F. + A.	A.	
Disease stage:														
• I	51 (11.5)	30 (13.5)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
• II	137 (30.9)	68 (30.6)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
• III	72 (16.2)	39 (17.6)	1 (0.3)	3 (0.9)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
• IV	138 (31.1)	72 (32.4)	333 (99.7)	331 (99.1)	NR	NR	NR	NR	202 (88)	200 (86)	NR	NR	245 (95)	242 (94.5)
• Unknown	36 (8.1)	1 (0.5)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Site of metastasis:														
• Bone only	103 (23.2)	48 (21.6)	69 (20.1)	78 (23.4)	70 (21.3)	39 (23.6)	81 (24)	78 (23)	24 (10)	24 (10)	76 (22.0)	75 (21.5)	63 (24.4)	71 (27.7)
• Visceral	214 (48.2)	110 (49.5)	197 (59.0)	196 (59.0)	172 (52.4)	89 (53.9)	193 (58)	188 (56)	135 (59)	119 (51)	167 (48.4)	181 (51.9)	134 (51.9)	124 (48.4)
• Non- visceral	230 (51.8)	112 (50.5)	NR	NR	NR	NR	NR	NR	60 (26)	81 (35)	102 (29.6)	93 (26.6)	195 (28.1)	NR
• Lymph nodes	NR	NR	133 (39.8)	123 (36.8)	NR	NR	142 (42)	158 (47)	NR	NR	NR	NR	NR	NR
• Other	NR	NR	45 (12.9)	33 (9.9)	86 (26.2)	37 (22.4)	8 (2)	8(2)	11 (4)	8 (4)	NR	NR	1 (0.4)	1 (0.4)
Measurable disease:														
• Yes	NR	NR	NR	NR	267 (81.4)	130 (78.8)	NR	NR	193 (84)	196 (84)	188 (54.5)	188 (53.9)	129 (50.0)	113 (44.1)
• No	NR	NR	NR	NR	61 (18.6)	35 (21.2)	NR	NR	NR	NR	157 (45.5)	161 (46.1)	129 (50.0)	143 (55.9)
Disease-free interval:														
• <i>De novo</i>	167 (37.6)	81 (36.5)	114 (34.1)	113 (33.8)	NR	NR	136 (41)	134 (40)	NR	NR	NR	NR	NR	NR
• ≤12 months	99 (22.3)	48 (21.6)	4 (1.2)	10 (3.0)	NR	NR	23 (7)	13 (4)	NR	NR	NR	NR	14 (5.4)	18 (7.0)
• >12 months	178 (40.1)	93 (41.9)	216 (64.7)	210 (62.9)	NR	NR	176 (53)	190 (56)	NR	NR	NR	NR	85 (32.9)	78 (30.5)
• Unknown	NR	NR	NR	1 (0.3)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

Table 3. *Contd.*

Characteristics	PALOMA-2, n (%)		MONALEESA-2, n (%)		MONARCH-3, n (%)		MONALEESA-7, n (%)		FALCON <sup>a</sup> , n (%)		SWOG <sup>b</sup> , n (%)		FACT4, n (%)	
	L. + Palb.	L.	L. + Rib.	L.	L or A. + Abem.	L. or A.	Rib. + T or nAls	T or nAls	F.	A.	F. + A.	A.	F. + A.	A.
No. of disease sites														
• 0	NR	NR	2 (0.6)	1 (0.3)	NR	NR	1 (<1)	NR	NR	NR	NR	NR	NR	NR
• 1	138 (31.1)	66 (29.7)	100 (29.9)	117 (35.0)	96 (29.3)	47 (28.5)	112 (33)	117 (35)	NR	NR	NR	NR	NR	NR
• 2	117 (26.4)	52 (23.4)	118 (35.3)	103 (30.8)	76 (23.2)	42 (25.5)	106 (32)	99 (29)	NR	NR	NR	NR	NR	NR
• 3	112 (25.2)	61 (27.5)	114 (34.1)	113 (33.8)	154 (47) <sup>2</sup>	75 (45.5) <sup>2</sup>	116 (35)	121 (36)	NR	NR	NR	NR	NR	NR
• ≥4	77 (17.3)	43 (19.4)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Prior chemotherapy:														
• Adjuvant	180 (40.5)	89 (40.1)	146 (43.7)	145 (43.4)	125 (38.1)	66 (40.0)	138 (41)	138 (41)	35 (15)	27 (12)	103 (29.9)	129 (37.0)	108 (41.9)	127 (49.6)
• Neoadjuvant	54 (12.2)	32 (14.4)	*	*	*	*	*	*	11 (5)	16 (7)	NR	NR	NR	NR
• Palliative	NR	NR	NR	NR	NR	NR	47 (14)	47 (14)	36 (16)	43 (19)	NR	NR	NR	NR
• None	NR	NR	NR	NR	203 (61.9)	99 (60.0)	150 (45)	152 (45)	NR	NR	242 (70.1)	220 (63.0)	NR	NR
Prior hormonal therapy:														
• Adjuvant	249 (56.1)	126 (56.8)	175 (52.4)	171 (51.2)	150 (45.7)	80 (48.5)	127 (38)	141 (42)	2 (1)	1 (<1)	139 (40.3)	141 (40.4)	180 (69.8)	168 (65.6)
• Neoadjuvant	NR	NR	*	*	*	*	208 (62)	196 (58)	NR	NR	206 (59.7)	208 (59.6)	NR	NR
• None	NR	NR	NR	NR	178 (54.3)	85 (51.5)	208 (62)	196 (58)	NR	NR	206 (59.7)	208 (59.6)	NR	NR
Type of adjuvant ET:														
• Tamoxifen	209 (47.1)	98 (44.1)	140 (41.9)	145 (43.4)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
• Anastrozole	56 (12.6)	29 (13.1)	47 (14.1)	42 (12.6)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
• Letrozole	36 (8.1)	16 (7.2)	34 (10.2)	25 (7.5)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
• Exemestane	30 (6.8)	13 (5.9)	19 (5.7)	25 (7.5)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
• Other	NR	NR	8 (2.4)	7 (2.1)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

<sup>a</sup> The experimental arm consisted of F 500 mg intramuscular injection; on days 0, 14, 28, then every 28 days thereafter. <sup>b</sup> The experimental arm consisted of F 500 mg on day 1 and 250 mg on days 14 and 28 and monthly thereafter. \* Patients included among those of the adjuvant setting. L: letrozole; Palb.: palbociclib; Rib: Ribociclib; A: anastrozole; Abe: abemaciclib; T: tamoxifen; nAls: non-steroidal aromatase inhibitors; F: fulvestrant; NR: not reported; ECOG: Eastern Cooperative Oncology Group; ER: estrogen receptor; PgR: progesteron receptor; +: positive.

#### 4. Discussion

HR+/HER2− BC is the most common subtype of this disease, representing approximately 60–70% of all breast tumors [1]. For many years, the sequential use of ET was the preferred approach in HR+ MBC patients due to its effectiveness and favorable toxicity profile [3,30,31]. The recent introduction of new combinations of ET plus CDK 4/6 or phosphatidylinositol 3-kinases (PI3K) inhibitors led to further clinical improvement in HR+ MBC patients [30,31]. Regarding the first-line setting, several randomized phase III clinical trials clearly demonstrated that the three highly selective CDK 4/6 inhibitors (palbociclib, ribociclib, abemaciclib) significantly improve ORR and CB and prolong PFS when combined with an AI or tamoxifen, both in pre- and post-menopausal women [12–15].

Although clinical outcomes are very similar for the three CDK 4/6 inhibitors and are not influenced by classic clinical and pathological factors, several differences were recognized concerning their toxicity profile and mechanism of action. In the patients who received the combination CDK 4/6 inhibitor plus ET, a higher incidence of hematologic adverse events occurred compared to control groups. The most common hematologic adverse events with grade 3 or 4 were neutropenia (43% vs. 1%), leucopenia (20% vs. 0.4%), anemia (5% vs. 1%), and thrombocytopenia (2% vs. 0.1%) [32]. Despite the high incidence of neutropenia reported in the RCTs, a higher incidence of febrile neutropenia was not recorded, which is evident just in 1.3% of patients. Unlike palbociclib and ribociclib, abemaciclib caused low-grade diarrhea and transaminase elevation, readily managed with conventional medications or dose reduction [17]. On the other hand, data suggest that abemaciclib has distinct single-agent activity at the molecular level, which could reflect its unique effects and toxicity profile [33]. For example, abemaciclib, but not ribociclib or pabociclib, is a potent inhibitor of kinases other than CDK 4/6, including CDK1/cyclin B, which appears to cause arrest in the G2 phase of the cell cycle, and CDK2/cyclin E/A, which is implicated in resistance to palbociclib. Whereas ribociclib and palbociclib induce cytostasis, and cells adapt to these drugs within 2–3 days of exposure, abemaciclib induces cell death and durably blocks cell proliferation. Abemaciclib is active even in pRB-deficient cells in which CDK 4/6 inhibition by palbociclib or ribociclib is completely ineffective [34]. In luminal tumors, some useful biomarkers are being studied to identify the best target treatment combined with anti-hormonal therapy and which can then determine the ideal choice for activity rather than toxicity. Recently, the *PI3K* mutation presented in progressive luminal tumors from the first-line treatment with AI showed a significant benefit in PFS when associated with F [35].

Against this background, knowing whether these three agents can be interchangeable remains an urgent unmet clinical need [21,32,36,37]. Thus, a better understanding of molecular differences is a relevant challenge since it could be informative for their right use in the clinical setting. Additionally, since there are no comparative data between CDK 4/6 inhibitors and F, the hormonal agent approved in endocrine-naïve patients with MBC for first-line setting, the identification of the most suitable HR+ MBC patients who can benefit most from the less toxic F-based-therapy is still a significant challenge [5,21]. Herein, we performed a meta-analysis, including only data from phase III RCTs available concerning the same clinical scenario. This approach allows the synthesis of a large body of evidence while retaining the benefits of randomization within each trial. Hence, we use this indirect comparison method to investigate whether F-based ET could still play a role in some specific subgroup of patients with HR+ MBC in the first-line setting.

In this meta-analysis, we found that CDK 4/6 inhibitors produced significant improvement in ORR, CB, and PFS in all patients with HR+ MBC in comparison with F-based therapies. Furthermore, these results were independent of age, race, performance status, disease site, prior chemotherapy, prior ET, disease-free interval after adjuvant treatment, menopausal status, type of CDK 4/6 inhibitor or expression of the progesterone receptor. Interestingly, significant PFS improvement in favor of CDK 4/6 inhibitors was observed even in patients with bone-only disease and in non-visceral disease. Due to the lack of convincing efficacy criteria to prefer one or the other CDK 4/6 inhibitor, the choice should rely on the toxicity profile. It must be remembered, however, that no direct comparison has ever been performed between the three CDK 4/6 inhibitors to allow an appropriate selection in the clinic.

Our meta-analysis has several limitations suggesting caution in interpreting the results. First, it is not based on individual patient data but was conducted considering the HR and 95% CIs of each study extracted. Moreover, for some subgroups, the HRs were not reported in all studies, particularly in F-based trials. In addition, the number of trials was relatively limited, preventing formal comparisons among all treatment strategies for each group. Second, and this is a well-known caveat of studies with F, not all the included studies with this compound used the now considered standard dose (500 mg monthly plus an additional dose of 500 mg for 15 days during the first month) [25,27]. The CONFIRM trial has revealed a superior activity of 500 mg when compared with 250 mg of F in terms of PFS and OS [6]. Therefore, the non-standard F dose used in some trials may partially bias our findings. Third, while the four RCTs [12–15] including CDK 4/6 inhibitors are rather homogenous in terms of inclusions criteria and patient characteristics, the greater heterogeneity observed in F-based studies [5,25,27] inevitably affected the pooled meta-analyses results. For instance, the line of therapy, metastatic sites, tumor burden, prior anti-estrogen drugs, and anti-estrogen treatment sequence, previous endocrine therapies sensitivity were also heterogeneous. Notably, F was more effective in endocrine-naïve patients without the visceral disease [5]. As demonstrated in Table 1, the FALCON trial exclusively included those patients who had never been exposed to ET, both in the metastatic and early settings. By contrast, a percentage ranging from 40 to 65% of patients included in the remaining trials were previously exposed to ET in the adjuvant or neoadjuvant settings. In addition, F efficacy in “bone-only” limited disease was not established in all studies [27]. Finally, for peri and premenopausal patients, we cannot rule out the possibility that the administered luteinizing hormone-releasing hormone analog (LHRHa) may affect the final estimation, as LHRHa itself was reported as an effective endocrine approach in breast cancer. Moreover, the limited number of peri or premenopausal patients in F-based studies makes a comparison between F and CDK 4/6 inhibitor ET strategies unreliable in this setting of patients. Specifically, while the FALCON and SWOG trials were enrolled merely post-menopausal patients, in the FACT trial, premenopausal patients were also included and were treated with GnRH<sub>a</sub>. Unfortunately, they only represented 3.1% of the experimental arm. Although previous reviews and meta-analyses of treatments in MBC patients have been conducted, many of these studies focus on population different from the current study. For example, Ayyagari et al. explored the safety and efficacy of only two CDK 4/6 inhibitors (palbociclib and ribociclib) in postmenopausal women after progression on a non-steroidal AI [35]. Ding et al. also conducted a similar NMA, focusing on the results of all three CDK4/6 inhibitors in phase II and III trials both in the first and second lines; however, the analysis did not include a direct comparison with F in first-line treatment [32]. Finally, El Rassy et al. investigated which CDK4/6 inhibitor was more effective in patients with luminal breast cancer, but their study was limited by the small sample size and lack of studies on F (1 included) [36]. Therefore, our study specifically focused on the results of phase III studies in the first-line setting and comprised an indirect comparison with first-line F. In addition, the present meta-analysis includes recently published data that were not included in previous reviews, in particular, the results of MONALEESA-7 on premenopausal women [32,36,37]. Despite the differences in study design and specific limitations, the results of our and previous NMAs are consistent in reporting the improved clinical outcomes obtained with CDK4/6 inhibitors compared with monotherapy [32,36,37].

## 5. Conclusions

In conclusion, the results of this meta-analysis confirm that CDK 4/6 inhibitors have similar efficacy when associated with an AI in the first-line treatment of HR+ MBC, and are superior to either F or AI monotherapy, regardless of any other patients or tumor characteristics. Though all CDK 4/6 inhibitors resulted associated with similar outcomes, the differences in toxicity profile, drug interactions, and patient preferences seem to be the main factors to be considered in the clinical decision-making process. Interestingly, CDK 4/6 inhibitors with AI resulted in more effective than F-based therapies even in patients with the bone-limited disease and, what is more, in patients without visceral disease involvement. Hence, the use of F in the first-line setting is destined to be abandoned as a single agent,

and its right place in HR+ MBC patients management needs to be urgently refined. Based on preclinical and early clinical trial results, its mechanism of action and pharmacokinetic properties make it an ideal backbone for combination therapies contributing to overcome or delaying endocrine resistance. Rational combinations with other therapies, such as *PI3K* inhibitors, HER2-directed therapies, and immunotherapy, are being explored. The emerging data also suggest a potential use of CDK4/6-targeted approaches in neoadjuvant settings (Supplementary Table S1). Different clinical trials are also ongoing to assess the safety and efficacy of CDK4/6 inhibitors alone or in combination with chemotherapy in different groups of patients. These trials, together with future comparative studies and biomarker analyses, are indispensable to better select patients who derive the greatest benefit from a specific class of CDK 4/6 inhibitors.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/11/11/1661/s1>, Table S1: The most important phase II or III ongoing clinical trials investigating CDK 4/6 inhibitors in breast cancer.

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Article

# Therapy Landscape in Patients with Metastatic HER2-Positive Breast Cancer: Data from the PRAEGNANT Real-World Breast Cancer Registry

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**Abstract:** This study presents comprehensive real-world data on the use of anti-human epidermal growth factor receptor 2 (HER2) therapies in patients with HER2-positive metastatic breast cancer (MBC). Specifically, it describes therapy patterns with trastuzumab (H), pertuzumab + trastuzumab (PH), lapatinib (L), and trastuzumab emtansine (T-DM1). The PRAEGNANT study is a real-time, real-world registry for MBC patients. All therapy lines are documented. This analysis describes the utilization of anti-HER2 therapies as well as therapy sequences. Among 1936 patients in PRAEGNANT, 451 were HER2-positive (23.3%). In the analysis set (417 patients), 53% of whom were included in PRAEGNANT in the first-line setting, 241 were treated with H, 237 with PH, 85 with L, and 125 with T-DM1 during the course of their therapies. The sequence PH → T-DM1 was administered in 51 patients. Higher Eastern Cooperative Oncology Group (ECOG) scores, negative hormone receptor status, and visceral or brain metastases were associated with more frequent use of this therapy sequence. Most patients received T-DM1 after treatment with pertuzumab. Both novel therapies (PH and T-DM1) are utilized in a high proportion of HER2-positive breast cancer patients. As most patients receive T-DM1 after PH, real-world data may help to clarify whether the efficacy of this sequence is similar to that in the approval study.

**Keywords:** advanced breast cancer; metastatic; chemotherapy; antihormone therapy; HER2 c-erbB2; HER2/neu; trastuzumab; pertuzumab; T-DM1; lapatinib

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## 1. Introduction

Overexpression of human epidermal growth factor receptor 2 (HER2), or amplification of the *HER2* gene, is seen in approximately 15–25% of breast cancer (BC) patients [1]. Since the discovery in the late 1980s of HER2 amplifications and their prognostic relevance [2], treatment for HER2-positive BC in this subgroup of patients has greatly improved [3–6]. Adding the monoclonal anti-HER2 antibody trastuzumab to standard chemotherapy resulted in a significant improvement in the progression-free survival (PFS) and overall survival (OS) in patients with metastatic HER2-positive BC [7]. These results led to the approval of trastuzumab for the treatment of HER2-positive metastatic BC.

Later, the dual tyrosine kinase inhibitor lapatinib was also analyzed in this group of patients. Women whose cancers had progressed after treatment with an anthracycline, a taxane, and trastuzumab were randomly assigned to therapy with capecitabine plus lapatinib or capecitabine alone. In contrast to the monotherapy, the combination treatment led to a significantly longer PFS. Therefore, lapatinib also became the standard of care in the early 2000s [8,9].

The CLEOPATRA study demonstrated an additional improvement in survival outcomes in treatment-naïve (chemotherapy and biological therapy, one endocrine treatment was allowed) HER2-positive patients with metastatic BC. Patients who were receiving docetaxel and dual HER2 blockade with trastuzumab plus pertuzumab, another monoclonal HER2 antibody, were compared with patients receiving docetaxel plus trastuzumab alone. The improved survival results led to the approval of pertuzumab for the first-line treatment setting. The enrolled patients were allowed to have had (neo)adjuvant chemotherapy with or without trastuzumab. However, the observed benefit

of the addition of palliative pertuzumab was independent of any previous (neo)adjuvant treatment with trastuzumab [10,11].

Another HER2-targeted approved drug is trastuzumab emtansine (T-DM1), which was designed as an antibody–drug conjugate to target specifically HER2-enriched tumor cells, and in this way, reduce side effects in nontargeted tissue. In the EMILIA trial, the efficacy of T-DM1 was analyzed in women with HER2-positive advanced or metastatic disease who had previously been treated with a taxane and trastuzumab in the advanced therapy setting and were randomly assigned to second-line or further treatment with T-DM1 versus capecitabine plus lapatinib. It was found that T-DM1 was clearly superior with regard to survival outcomes in comparison with the control arm [12].

After the approval of T-DM1 and pertuzumab, the question arose of whether a combination of the two might result in an additional benefit. However, the MARIANNE study showed in first-line HER2-positive metastatic BC patients that neither T-DM1 alone nor T-DM1 in combination with pertuzumab improved the PFS in comparison with trastuzumab plus a taxane [13]. Although the reason for this remains unclear, there are cell line data that suggest that the correct therapy sequence for the drugs might have an influence on the treatment response [14].

Moreover, novel substances are also being investigated to further improve outcomes for patients. For instance, neratinib, another tyrosine kinase inhibitor, was recently approved for the adjuvant treatment of patients with HER2-positive early BC, due to its significant improvement of five-year disease-free survival (DFS) [15], and is currently also being analyzed in the metastatic setting. Afatinib, however, did not show any improvement in the outcomes for patients with metastatic BC in comparison with trastuzumab [16]. Margetuximab has now made available a third novel HER2 antibody that appears to enhance antibody-dependent cellular toxicity (ADCC), while at the same time being well-tolerated [17]. Its efficacy and safety are currently being investigated in the phase III SOPHIA trial in patients with HER2-positive metastatic BC who were previously treated with trastuzumab, pertuzumab, and T-DM1 [14].

As more and more therapy options become available, it is possible that treatment sequences may no longer be following the same inclusion and exclusion criteria as those that applied in the respective clinical trials. Understanding current therapy practice may be helpful for estimating the extent to which results from clinical trials can be generalized for specific patient populations. Therefore, the objective of this study is to describe comprehensive real-world evidence on the use of trastuzumab, pertuzumab, lapatinib, and T-DM1 in first-line treatment in the metastatic setting.

## 2. Patients and Methods

### 2.1. The PRAEGNANT Research Network

The PRAEGNANT study (Prospective Academic Translational Research Network for the Optimization of the Oncological Health Care Quality in the Adjuvant and Advanced/Metastatic Setting; NCT02338167 [18]) is an ongoing, prospective BC registry with a documentation system similar to that of a clinical trial. The aims of PRAEGNANT are to assess treatment patterns and quality of life, and to identify patients who may be eligible for clinical trials or specific targeted treatments [18–21]. Patients can be included at any point in time during the course of their disease. All of the patients included in the present study provided informed consent, and the study was approved by the relevant ethics committees.

### 2.2. Patients

A total of 2379 patients with advanced or metastatic BC were registered in the PRAEGNANT study between July 2014 and March 2018 at 52 study sites. Patients were excluded in the following hierarchical order: 39 patients were excluded due to unknown HER2 status, as well as 53 patients due to unknown hormone receptor status. In 138 patients, the date of the first diagnosis of a metastasis or their birth date was missing. Therefore, these patients also had to be excluded. Male patients ( $n = 20$ )

were also not included in the analysis. Treatment information was not available for an additional 193 women, leaving 1936 patients for whom the above-mentioned data were known. A total of 451 of these women had HER2-positive tumors (Figure 1). For analysis, patients were divided into distinct patient groups based on the documentation status concerning the therapy lines. Group 1 is defined as the patient population for which only the first therapy line is documented. Group 2 is the population for which the first and the second therapy lines are documented. Group 3 is the patient population for which only the first, seco, nd and third therapy lines are documented. Group 4 is the patient population for which at least the first to the forth therapy lines are documented. Each group cannot be part of the other groups. These groups are the natural consequence of patients being treated with more or less therapy lines in the metastatic setting.

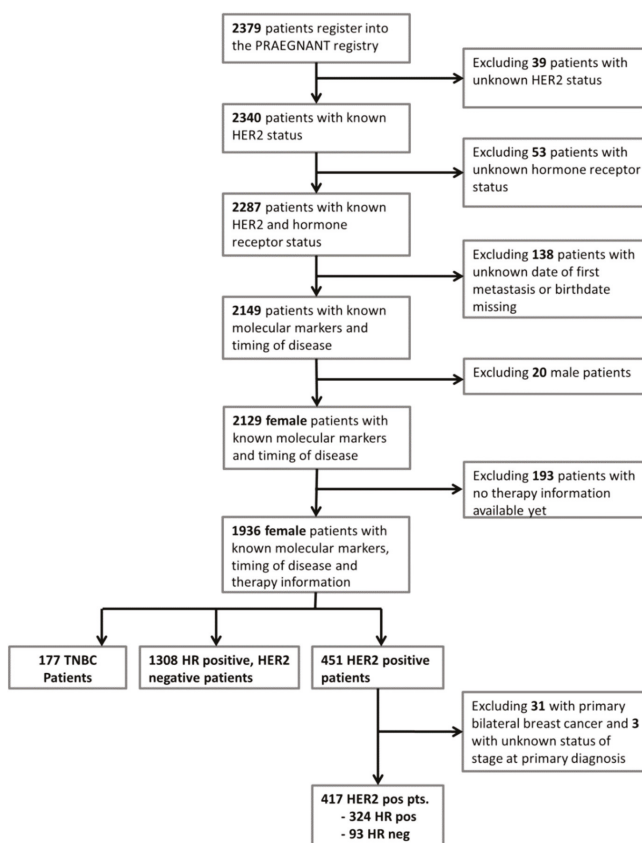


Figure 1. Patient flow chart and patient selection.

### 2.3. Data Collection

The data were collected by trained staff and documented in an electronic case report form [18]. The data were monitored using automated plausibility checks and on-site monitoring. Data that are not usually documented as part of routine clinical work are collected prospectively using structured questionnaires completed on paper. These consist of epidemiological data, such as family history, cancer risk factors, quality of life, nutrition and lifestyle items, and psychological health. Supplementary Table S1 provides an overview of the collected data.

#### 2.4. Definition of Hormone Receptors, HER2 Status and Grading

The definition of hormone receptors, HER2 status, and grading was described previously [19]. Briefly, data about estrogen receptor status, progesterone receptor status, HER2 status, and grading were obtained for documentation purposes for each tumor that had been biopsied. Therefore, there could be several possible sources (right breast, left breast, local recurrence, metastatic site). Biomarker status for ER, PR, and HER2 were determined as follows: If a biomarker assessment of the metastatic site was available, this receptor status was used for the analysis. If there was no information available for metastases, the latest biomarker results from the primary tumor were used. Additionally, all patients who received estrogen therapy in the metastatic setting were assumed to be HR-positive, and all patients who had ever received anti-HER2 therapy were assumed to be HER2-positive. There was no central review of biomarkers. The study protocol recommended assessing ER and PR status as positive if  $\geq 1\%$  was stained. A positive HER2 status required an immunohistochemistry score of 3+ or positive fluorescence in situ hybridization/competitive in situ hybridization (FISH/CISH).

#### 2.5. Statistical Considerations

The analysis and reporting of treatments are descriptive. The total number of treatments for each of the following four therapy lines are provided: Trastuzumab (H), trastuzumab and pertuzumab (PH), lapatinib (L), and trastuzumab emtansine (T-DM1). It was also analyzed whether patients who had already completed a specific number of therapy lines (1–4) received these four anti-HER2 therapies in any therapy line. For this purpose, the patients were categorized into four distinct groups, namely: Patients for whom only the first therapy line was documented, patients for whom the first two therapy lines were documented, patients for whom the first three therapy lines were documented, and patients for whom at least the first four therapy lines were documented. Similarly, the frequencies of usage of the PH  $\rightarrow$  T-DM1 and T-DM1  $\rightarrow$  PH therapy sequences were analyzed, regardless of whether these therapies followed each other directly.

It was also analyzed whether the patients' characteristics were associated with the frequency of utilization of the PH  $\rightarrow$  T-DM1 sequence in the first four therapy lines, again regardless of whether these therapies followed each other directly.

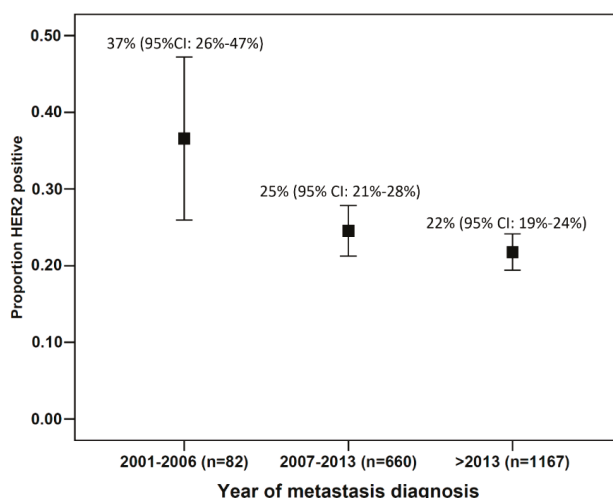
With regard to the year of therapy, the patients were also categorized in relation to their first four therapy lines. The first group consisted of patients who completed all documented therapy lines before 2013, the second group had to have had at least one treatment administered before 2013 and one in 2013 or after 2013. In the last group, all patients had to have received all treatments after 2013.

Calculations were performed using IBM SPSS Statistics, version 24 (Armonk, New York, NY, USA: IBM Corporation).

### 3. Results

#### 3.1. Patient and Disease Characteristics

A total of 451 (23.3%) patients in the registry had HER2-positive metastatic breast cancer. Figure 2 shows the frequency of HER2-positive metastatic breast cancer patients over the years. Although the HER2 status was positive in 37% (95% CI: 26–47%) of all patients with metastases who were treated up to 2006, HER2 positivity was seen in 25% (95% CI: 21–28%) and 22% (95% CI: 19–24%) of patients diagnosed with metastases in 2007–2013 and after 2013, respectively (Figure 2). For further analyses, patients with bilateral breast cancer at diagnosis and those with missing information about the stage at initial diagnosis were excluded. The final study population comprised 417 patients, 324 of whom were hormone receptor-positive and 93 hormone receptor-negative (Figure 1).



**Figure 2.** Proportion of human epidermal growth factor receptor 2 (HER2)-positive patients with 95% confidence intervals relative to the year in which the metastases were diagnosed.

The characteristics of the patients and diseases are listed in Table 1. Most patients entered the study in the first-line setting, had an Eastern Cooperative Oncology Group (ECOG) score of 0, and had visceral metastases. Approximately 40% of the patients had metastases at the time of diagnosis.

**Table 1.** Patient characteristics at baseline.

Characteristic	n or Mean	% or SD
Age at study entry	57.9	13.0
BMI	26.0	5.3
Time from diagnosis to metastasis (days)	1177.8	1743.0
Therapy situation at study entry		
First-line	223	53.5
Second-line	70	16.8
Third-line	53	12.7
Fourth-line	26	6.2
Fifth-line and higher	34	8.2
Therapy situation at database closure		
First-line	171	41.0
Second-line	82	19.7
Third-line	47	11.3
Fourth-line	17	4.1
Fifth-line and higher	59	14.1
Hormone receptor status		
Negative	93	22.3
Positive	324	77.7
ECOG		
0	196	47.0
1	155	37.2
2	35	8.4
3	12	2.9
4	2	0.4

Table 1. Cont.

Characteristic	n or Mean	% or SD
Metastasis site at study entry		
Brain <sup>a</sup>	79	18.9
Visceral <sup>b</sup>	222	53.2
Bone only	58	13.9
Other <sup>c</sup>	50	12.0
Metastatic at time of diagnosis		
No	244	58.5
Yes	173	41.5

BMI (body mass index) ECOG (Eastern Cooperative Oncology Group) (score); SD (standard deviation). <sup>a</sup> Patients included in the “brain” group were allowed to have metastases at any other site. <sup>b</sup> Patients included in the “visceral” group were allowed to have metastases at any other site except the brain. <sup>c</sup> Patients included in this group were not allowed to have any brain, visceral, or bone metastases.

### 3.2. Therapies

Across all of the therapy lines (1 to 9+), 241 of the 417 patients were treated with H without additional anti-HER2 therapy, 237 with PH, 85 with L, and 125 with T-DM1. The respective figures up to therapy line four are 236 (H), 220 (PH), 79 (L), and 108 (T-DM1) patients.

Table 2 shows patterns of therapy utilization relative to patient groups with first-line therapy documented, with first- and second-line therapy documented, with first- to third-line therapy documented, and with first- to fourth-line therapy documented, relative to the treatment period. Trastuzumab, either as a single anti-HER2 therapy or together with pertuzumab, was already administered in over 80% of the patients for whom only the first-line was documented. PH utilization increased across the different time periods, with approximately 60–70% of all patients already receiving this treatment as first-line therapy. T-DM1 utilization also increased across the time periods, although patients with a larger number of documented therapy lines had a higher frequency (approximately 52% of patients had four therapy lines documented and approximately 33% of patients had only two therapy lines documented). Lapatinib use did not change across the time periods and was mainly administered in later therapy lines (Table 2).

**Table 2.** Frequencies of patients who received the respective treatments. The patients are categorized here into four mutually exclusive (distinct) patient groups according to the number of documented therapy lines. The percentages of patients treated are marked in bold. The numbers and percentages of treated patients refer to the cumulative number of patients treated up to the highest documented therapy line. For example, in the group of patients with three therapy lines documented and treated after 2013, 33 patients have been treated with trastuzumab in one of the first three therapy lines.

Therapy	Patients Treated Before 2012		Patients Treated Crossing 2013		Patients Treated After 2013	
	Not Treated	Treated	Not Treated	Treated	Not Treated	Treated
Trastuzumab (H)						
treatments in patient group 1 <sup>a</sup>	4 (19)	17 ( <b>80.9</b> )	0 (0)	6 ( <b>100</b> )	28 (20.4)	109 ( <b>79.5</b> )
treatments in patient group 2 <sup>b</sup>	0 (0)	6 ( <b>100</b> )	2 (13.3)	13 ( <b>86.6</b> )	11 (15)	62 ( <b>84.9</b> )
treatments in patient group 3 <sup>c</sup>	0 (0)	4 ( <b>100</b> )	3 (15.7)	16 ( <b>84.2</b> )	6 (15.3)	33 ( <b>84.6</b> )
treatments in patient group 4 <sup>d</sup>	3 (20)	12 ( <b>80</b> )	8 (18.1)	36 ( <b>81.8</b> )	7 (18.4)	31 ( <b>81.5</b> )
Trastuzumab + pertuzumab (PH)						
treatments in patient group 1 <sup>a</sup>	19 (90.4)	2 ( <b>9.5</b> )	3 (50)	3 ( <b>50</b> )	51 (37.2)	86 ( <b>62.7</b> )
treatments in patient group 2 <sup>b</sup>	6 (100)	0 (0)	11 (73.3)	4 ( <b>26.6</b> )	21 (28.7)	52 ( <b>71.2</b> )
treatments in patient group 3 <sup>c</sup>	4 (100)	0 (0)	13 (68.4)	6 ( <b>31.5</b> )	12 (30.7)	27 ( <b>69.2</b> )
treatments in patient group 4 <sup>d</sup>	15 (100)	0 (0)	28 (63.6)	16 ( <b>36.3</b> )	14 (36.8)	24 ( <b>63.1</b> )



Table 2. Cont.

Therapy	Patients Treated Before 2012		Patients Treated Crossing 2013		Patients Treated After 2013	
	Not Treated	Treated	Not Treated	Treated	Not Treated	Treated
Lapatinib (L)						
treatments in patient group 1 <sup>a</sup>	20 (95.2)	1 (4.7)	5 (83.3)	1 (16.6)	134 (97.8)	3 (2.1)
treatments in patient group 2 <sup>b</sup>	6 (100)	0 (0)	12 (80)	3 (20)	65 (89)	8 (10.9)
treatments in patient group 3 <sup>c</sup>	3 (75)	1 (25)	15 (78.9)	4 (21)	30 (76.9)	9 (23)
treatments in patient group 4 <sup>d</sup>	8 (53.3)	7 (46.6)	22 (50)	22 (50)	18 (47.3)	20 (52.6)
Trastuzumab emtansine (T-DM1)						
treatments in patient group 1 <sup>a</sup>	21 (100)	0 (0)	6 (100)	0 (0)	131 (95.6)	6 (4.3)
treatments in patient group 2 <sup>b</sup>	6 (100)	0 (0)	10 (66.6)	5 (33.3)	49 (67.1)	24 (32.8)
treatments in patient group 3 <sup>c</sup>	4 (100)	0 (0)	8 (42.1)	11 (57.8)	21 (53.8)	18 (46.1)
treatments in patient group 4 <sup>d</sup>	14 (93.3)	1 (6.6)	21 (47.7)	23 (52.2)	18 (47.3)	20 (52.6)

<sup>a</sup> Group 1 is the patient population for which only the 1st therapy line is documented. These patients are not part of groups 2–4; <sup>b</sup> Group 2 is the patient population for which only the 1st and the 2nd therapy lines are documented. These patients are not part of the other groups. <sup>c</sup> Group 3 is the patient population for which only the 1st, 2nd and 3rd therapy lines are documented. These patients are not part of the other groups. <sup>d</sup> Group 4 is the patient population for which the 1st to the 4th therapy lines are documented. These patients are not part of the other groups.

The sequence of PH followed by T-DM1 (PH → T-DM1) was administered in 51 patients throughout all therapy lines and in 50 patients in lines one to four. Of those 50 patients, 48 patients were treated with the combination of PH and chemotherapy, and two with the combination of PH and endocrine therapy. Eleven patients received a sequence of T-DM1 → PH, eight of whom were treated within the first four therapy lines. This is equivalent to a utilization rate of PH → T-DM1 up to therapy line four after approval of about 42%, with an increase of approximately 10% from lines two to four per therapy line (Table 3).

**Table 3.** Frequencies of patients who were treated with the respective treatment sequences, irrespective of whether the sequences were administered directly after each other. The patients are categorized into four mutually exclusive patient groups according to the numbers of documented therapy lines. The percentages of patients treated are marked in bold.

Therapy	Patients Treated Before 2012		Patients Treated Crossing 2012		Patients Treated After 2012	
	Not Treated	Treated	Not Treated	Treated	Not Treated	Treated
Pertuzumab/trastuzumab → trastuzumab emtansine (PH → T-DM1)						
treatments in patient group 1 <sup>a</sup>	21 (100)	0 (0)	6 (100)	0 (0)	137 (100)	0 (0)
treatments in patient group 2 <sup>b</sup>	6 (100)	0 (0)	14 (93.3)	1 (6.6)	59 (80.8)	14 (19.1)
treatments in patient group 3 <sup>c</sup>	4 (100)	0 (0)	17 (89.4)	2 (10.5)	27 (69.2)	12 (30.7)
treatments in patient group 4 <sup>d</sup>	15 (100)	0 (0)	39 (88.6)	5 (11.3)	22 (57.8)	16 (42.1)
Trastuzumab emtansine → pertuzumab/trastuzumab (T-DM1 → PH)						
treatments in patient group 1 <sup>a</sup>	21 (100)	0 (0)	6 (100)	0 (0)	136 (99.2)	1 (0.7)
treatments in patient group 2 <sup>b</sup>	6 (100)	0 (0)	15 (100)	0 (0)	73 (100)	0 (0)
treatments in patient group 3 <sup>c</sup>	4 (100)	0 (0)	18 (94.7)	1 (5.2)	38 (97.4)	1 (2.5)
treatments in patient group 4 <sup>d</sup>	15 (100)	0 (0)	41 (93.1)	3 (6.8)	36 (94.7)	2 (5.2)

<sup>a</sup> Group 1 is the patient population for which only the 1st therapy line is documented. These patients are not part of groups 2–4; <sup>b</sup> Group 2 is the patient population for which only the 1st and the 2nd therapy lines are documented. These patients are not part of the other groups. <sup>c</sup> Group 3 is the patient population for which only the 1st, 2nd and 3rd therapy lines are documented. These patients are not part of the other groups. <sup>d</sup> Group 4 is the patient population for which the 1st to the 4th therapy lines are documented. These patients are not part of the other groups.

### 3.3. Predictors of the Use of a Therapy Sequence of PH Followed by T-DM1

Several patient and disease characteristics were analyzed in relation to their influence on the utilization of the therapy sequence PH → T-DM1 (Table 4). Age, grading, and stage at the initial diagnosis did not have any influence. Patients with higher ECOG scores appeared to be treated with this sequence more often, as well as patients with brain or visceral metastases. One patient with metastases only in the bone was treated with PH → T-DM1. Patients with a positive hormone receptor status were less frequently treated with the PH → T-DM1 sequence. Although only 23.7% of patients

with a positive hormone receptor status received this sequence up to therapy line four, hormone receptor–negative patients were treated with this sequence in about 44% of cases.

**Table 4.** Frequency of patients who received the treatment sequence pertuzumab/trastuzumab → trastuzumab emtansine (PH → T-DM1), irrespective of whether the sequences were administered directly after each other. All patients who had at least two documented therapy lines and in whom all treatments started after 2013 are included.

Characteristic	PH → T-DM1	
	No	Yes
Age		
<50	28 (65.1)	15 (34.9)
50–65	53 (72.6)	20 (27.4)
>65	27 (79.4)	7 (20.6)
Eastern Cooperative Oncology Group (ECOG) score		
0	59 (79.7)	15 (20.3)
1	34 (63.0)	20 (37.0)
2	6 (60.0)	4 (40.0)
3	5 (100)	0 (0)
Metastasis site at study entry		
Brain <sup>a</sup>	14 (58.3)	10 (41.7)
Visceral <sup>b</sup>	56 (68.3)	23 (31.7)
Bone only <sup>c</sup>	16 (94.1)	1 (5.9)
Other <sup>d</sup>	19 (82.6)	4 (14.7)
Hormone receptor status		
Negative	18 (56.3)	14 (43.8)
Positive	90 (76.3)	28 (23.7)
Grade		
1	2 (100)	0 (0)
2	45 (78.9)	12 (21.1)
3	50 (64.1)	28 (35.9)
Primary metastatic		
No	68 (73.9)	24 (26.1)
Yes	40 (69.0)	42 (28.0)

<sup>a</sup> Patients included in the “brain” group were allowed to have metastases at any other site. <sup>b</sup> Patients included in the “visceral” group were allowed to have metastases at any other site except the brain. <sup>c</sup> Patients included in this group were not allowed to have any brain, visceral, or bone metastases.

#### 4. Discussion

This analysis of a cohort from a real-world breast cancer registry shows how frequently anti-HER2 therapies are used. Although most patients received trastuzumab, the percentage of patients who received pertuzumab and trastuzumab, lapatinib, or T-DM1 was clearly lower. Most of the trastuzumab and pertuzumab therapies were administered in the first-line setting, but TDM-1 was administered in most cases between the second and fourth lines, and lapatinib more often in the third- and fourth-line setting. The sequence of TDM-1 after trastuzumab and pertuzumab was administered in up to 40% of patients with four therapy lines, while the sequence T-DM1 followed by pertuzumab was only administered in about 5% of the patients.

The analysis shows that HER2-positive metastatic breast cancer is a subgroup with a clinically relevant frequency. The frequency of HER2-positive patients in the present cohort of metastatic breast cancer patients was 23.3%, a rate similar to the initially described frequencies of 25–30% in primary breast cancer before the introduction of anti-HER2 therapies [2,22]. The frequency of triple-negative

breast cancer was much lower in this cohort, at 9.1% of all cases. Looking at HER2 positivity over the years, it seems that HER2 positivity decreased over time. One possible explanation could be the introduction of trastuzumab in the adjuvant setting [23–25], possibly reducing the number of HER2 positive patients, who would develop a metastasis at a later timepoint. However, it should be noted that the PRAEGNANT study has been registering patients since 2014, and data from before that year are purely descriptive. Therefore, this trend might be the result of a bias.

Pertuzumab and T-DM1 were approved in Germany in 2013. These therapies were thus inevitably not prevalent in the cohort before that time. Few patients were treated in clinical trials before that, and it can be clearly seen that the use of pertuzumab and trastuzumab during the first four therapy lines increased from 27–36% around 2013 to 63–71% after 2013. Most of these treatments were administered as first-line therapy, which is in accordance with the current national therapy guidelines [26]. T-DM1, which is administered after tumor progression in the metastatic setting, was already used in 23–58% of patients around 2013 and continued to be administered in 33–53% of patients after 2013. This therapy pattern also matched the current national therapy guidelines [26]. Most patients received T-DM1 after pertuzumab—a sequence that is under discussion, since at the time when the EMILIA study was conducted, only previous trastuzumab therapy was available [12]. A recent retrospective study did not show any differences in the prognosis when patients who had been treated with T-DM1 after pertuzumab were compared with patients who had not received previous T-DM1 treatment [27]. However, analyses of differences between subgroups with earlier and later therapy lines in which T-DM1 was administered were inconclusive [27]. The use of lapatinib did not change much over time in the first four therapy lines, despite the introduction of pertuzumab and T-DM1.

With regard to possible predictive factors that may have influenced physicians in deciding to treat patients with the pertuzumab–trastuzumab sequence, it appears that patients with more advanced disease or a less favorable prognosis were more likely to be treated with the PH → T-DM1 therapy sequence. Parameters that were associated with a higher frequency of PH → T-DM1 use were poorer ECOG scores, brain and visceral metastases, negative hormone receptor status, and higher grading. The higher frequency of PH → T-DM1 therapy in patients with brain metastases could be the enrichment of patients with brain metastases in patients treated with anti-HER2 therapies, while in patients with positive hormone receptor status, the avoidance of chemotherapy could be a motivation behind not giving an anti-HER2 directed therapy. With regard to visceral metastasis, its more frequent use in patients with visceral metastases could be explained with the possible need for an effective therapy regimen including anti-HER2 treatments and chemotherapy. Moreover, in exploratory subgroup analyses of both studies, CLEOPATRA and EMILIA patients with visceral metastases had a larger benefit from PH or T-DM1 than the patients treated with the respective comparator therapies [10–12].

To the best of our knowledge, no comparable data concerning this healthcare research question was previously published. Hormone receptor status in particular appears to be of special interest, since a desire to avoid chemotherapy in this patient group is a possible reason why specific treatment regimens are not administered.

This study has several strengths and limitations. Although the PRAEGNANT breast cancer registry has registered more than 2300 patients with metastatic breast cancer, only 451 of the patients were HER2-positive. Although this is a large number in comparison with other publications reporting on prospective or retrospective cohorts, the sample size might be low for identifying treatment patterns and possible predictors of patient/tumor characteristics that are associated with specific therapy sequences and possible outcomes. Clinical cancer registries might be helpful for gathering data on larger patient populations [28], but the degree of detail in the information might be limited in such population-based registries. Data completeness and detail are certainly strengths in the PRAEGNANT registry, which documents therapy lines, side effects, progression, and mortality with an approach similar to that used in clinical trials. Another fact that needs to be taken into consideration when interpreting the data is that in real-world cohorts, not all patients enter the study or end the

study at comparable time points. Patients in the first-line setting may therefore be overrepresented, as patients die during the course of the disease, or may be lost to follow-up. An attempt was made to account for this by categorizing the patients into groups for which documentation for all therapy lines up to a specific line was available, with treatment utilization being reported for each of these groups separately.

## 5. Conclusions

In conclusion, the utilization of trastuzumab appeared to be sufficiently high in this cohort of patients with metastatic breast cancer. The utilization of the PH → T-DM1 sequence appeared to be rather low, and the reasons for this should be analyzed in future studies.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/11/1/10/s1>, Table S1: Data categories captured in the PRAEGNANT study.

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Article

# Cell-Free DNA Variant Sequencing Using CTC-Depleted Blood for Comprehensive Liquid Biopsy Testing in Metastatic Breast Cancer

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**Abstract:** Liquid biopsy analytes such as cell-free DNA (cfDNA) and circulating tumor cells (CTCs) exhibit great potential for personalized treatment. Since cfDNA and CTCs are considered to give additive information and blood specimens are limited, isolation of cfDNA and CTC in an “all from one tube” format is desired. We investigated whether cfDNA variant sequencing from CTC-depleted blood (CTC-depl. B; obtained after positive immunomagnetic isolation of CTCs (AdnaTest EMT-2/Stem Cell Select, QIAGEN)) impacts the results compared to cfDNA variant sequencing from matched whole blood (WB). Cell-free DNA was isolated using matched WB and CTC-depl. B from 17 hormone receptor positive/human epidermal growth factor receptor 2 negative (HR+/HER2−) metastatic breast cancer patients (QIAamp MinElute ccfDNA Kit, QIAGEN). Cell-free DNA libraries were constructed (customized QIAseq Targeted DNA Panel for Illumina, QIAGEN) with integrated unique molecular indices. Sequencing (on the NextSeq 550 platform, Illumina) and data analysis (Ingenuity Variant Analysis) were performed. RNA expression in CTCs was analyzed by multimarker quantitative PCR. Cell-free DNA concentration and size distribution in the matched plasma samples were not significantly different. Seventy percent of all variants were identical in matched WB and CTC-depl. B, but 115/125 variants were exclusively found in WB/CTC-depl. B. The number of detected variants per patient and the number of exclusively detected variants per patient in only one cfDNA source did not differ between the two matched cfDNA sources. Even the characteristics of the exclusively detected cfDNA variants in either WB or CTC-depl. B were comparable. Thus, cfDNA variants from matched WB and CTC-depl. B exhibited no relevant differences, and parallel isolation of cfDNA and CTCs from only 10 mL of blood in an “all from one tube” format was feasible. Matched cfDNA mutational and CTC transcriptional analyses might empower a comprehensive liquid biopsy analysis to enhance the identification of actionable targets for individual therapy strategies.

**Keywords:** metastatic breast cancer; liquid biopsy; cell-free DNA; next-generation sequencing; circulating tumor cells



## 1. Introduction

Liquid biopsies harbor great potential for personalized treatment strategies and real-time monitoring approaches. In oncology, cell-free DNA (cfDNA) and, specifically, cell-free tumor DNA (ctDNA), defined by the presence of variants [1], as well as circulating tumor cells (CTCs), are powerful tools to describe tumor heterogeneity and clonal evolution [2].

High levels of ctDNA were significantly correlated with decreased overall survival (OS) in breast cancer (BC) [2] and, more specifically, *ESR1* cfDNA variants were associated with a shorter duration of endocrine treatment effectiveness in metastatic BC (MBC) [3]. Since patients with *ESR1* variants were described to benefit from fulvestrant rather than from exemestane, compared to patients without this alteration, *ESR1* variants also have the potential to tailor treatment regimens [4]. For disease monitoring, cfDNA concentration was shown to indicate impending relapse of primary BC earlier than any other imaging or blood-based strategy [5] and can predate treatment response changes [6,7].

The prognostic value of CTCs was first introduced for MBC patients more than a decade ago [8]. A decreased CTC count after treatment was significantly associated with increased progression-free survival (PFS) and OS [9]. Consequently, CTC count dynamics were proposed to be more suitable for monitoring than radiological imaging [10]. In addition to CTC counts, the expression profiles of CTCs were correlated with response evaluation criteria in solid tumors (RECIST) [11,12]. Positive results for therapy decisions in BC based on the CTC count, dynamics, or molecular characteristics, however, have until now not been convincingly examined in large randomized blinded clinical studies, but great efforts are underway to prove the predictive value of CTCs [2].

cfDNA and CTCs isolated from the same liquid biopsy specimen can enable comprehensive results if seen as providing complementary, rather than competitive information. CtDNA, released mostly passively, might present dying and probably therapy-sensitive cells [5]. On the other hand, CTCs are viable cells actively migrating into the circulation as potential seeds of metastasis and, therefore, possibly indicating minimal residual disease. It is not only possible to conduct mutational analysis of cfDNA and transcriptional analysis of CTCs using both analytes in parallel, but it was already described for multiple myeloma that sequencing of cfDNA and CTCs uncovered more mutations than analysis of either analyte alone [13]. Therefore, one might assume that parallel cfDNA and CTC analysis is complementary rather than competitive or even redundant [14].

Practically, however, many studies comparing cfDNA and CTCs used the same patient inclusion criteria, but different large cohorts for either cfDNA or CTC analysis [15,16]. There were a few publications analyzing both cfDNA and CTCs from the same patients, but the cohort sizes were quite small and the two analytes were obtained from blood samples taken at different time points [15,16]. Even if the study design considered the same time points for withdrawal, different preservative blood tubes, e.g., Streck and/or CellSave tubes, were mostly used [7,15,17–19]. In contrast, EDTA blood enabled parallel analysis of cfDNA and CTC from the same blood sample [20–23], but different blood aliquots were needed for both analyses, such that the required blood volume was around 20 mL [24]. Consequently, for appropriate comparability and consistency, the usage of the same blood sample with minimized volume drawn and stored/shipped under the same conditions for isolation of both analytes to reach an unbiased comprehensive liquid biopsy in an “all from one tube” format would be desirable.

We here (1) compared the quantity and characteristics of cfDNA variants isolated from whole blood (WB) and blood after positive immunomagnetic selection of CTCs (CTC-depleted (depl.) B), and (2) studied the heterogeneity of cfDNA variants and CTC overexpression signals in an hormone receptor positive/human epidermal growth factor receptor 2 negative (HR+/HER2–) MBC cohort.

## 2. Results

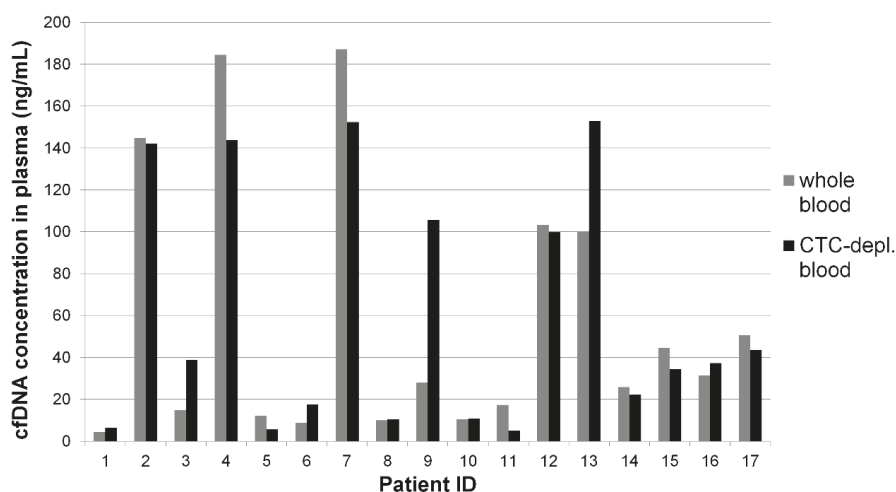
### 2.1. Patient Characteristics

The cohort was specified to only consist of patients with MBC with HR+/HER2– primary tumors. The majority of patients were more than 50 years old, and the median follow-up time was 68 months

(range: 12 to 322 months). Eleven of the seventeen patients were deceased at the time of analysis. At the time of blood draw, the majority of patients had secondary metastases, received more than three treatment lines, and exhibited progressive disease according to response evaluation criteria in solid tumors. All patient characteristics are listed in Table S1 (Supplementary Materials).

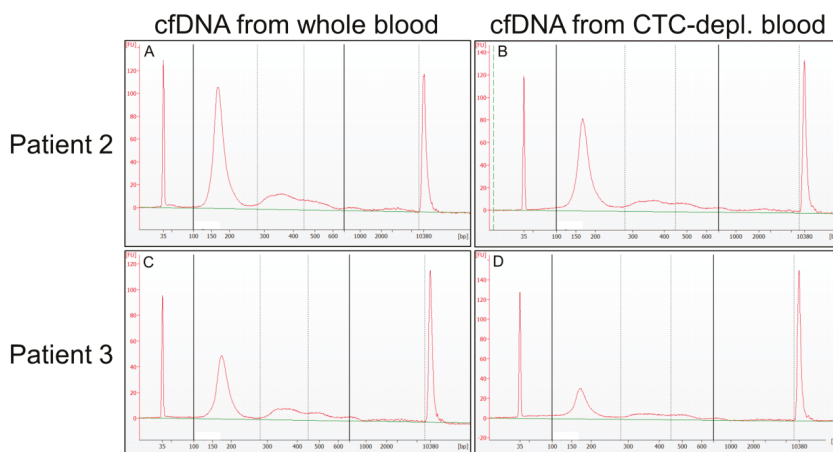
## 2.2. Cell-Free DNA Concentration and Fragmentation

The used volume of the matched plasma samples was identical and ranged from 2.8 mL to 6.0 mL (mean 4.4 mL). Cell-free DNA concentrations in the plasma of WB ranged from 4 ng/mL to 187 ng/mL (mean 58 ng/mL), while cfDNA concentration in plasma of CTC-depl. B ranged from 5 ng/mL to 153 ng/mL (mean 61 ng/mL). Wilcoxon signed-rank test considering all 17 matched samples revealed no significant difference of cfDNA concentration in the different plasma samples. However, in some matched samples, the cfDNA concentration differed greatly and, importantly, a high inter-individual variability was observed (Figure 1).



**Figure 1.** Inter-individual variability of cell-free DNA (cfDNA) concentration. Plasma from circulating tumor cell (CTC)-depleted (depl.)\_blood (by AdnaTest EMT-2/StemCell Select) and matched plasma from whole blood were used in the same volume for cfDNA isolation with a QIAamp MinElute ccfDNA Kit, and cfDNA was quantified using an Agilent High Sensitivity Chip (fragments between 100–700 bp).

Capillary electrophoresis using the Agilent High Sensitivity DNA Chip resolved the fragment length of the cfDNA. The majority of isolated cfDNA of all patients consisted of mononucleosomal cfDNA fragments (100–280 bp), but cfDNA fragments with a length of 280–450 bp/450–700 bp (di- or trinucleosomal DNA) were also detectable. Size distribution was comparable in cfDNA samples from WB and CTC-depl. B, as depicted for two exemplary patients (Figure 2). There was no difference in high-molecular-weight DNA (700–10,000 bp) in cfDNA eluates of the two plasma sources.

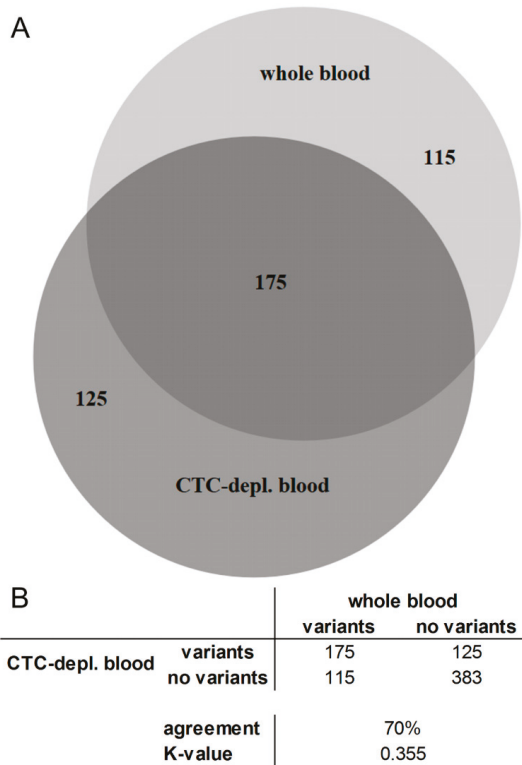


**Figure 2.** Size distribution of cfDNA. Matched cfDNA samples isolated from CTC-depl. blood (**B,D**; CTC isolation using AdnaTest EMT-2/StemCell Select) and from whole blood (**A,C**) of two exemplary patients (**A + B**; **C + D**) displayed a large mononucleosomal fraction and, in general, a similar size distribution without high-molecular-weight DNA (700–10,000 bp). Capillary electrophoresis was performed with an Agilent High Sensitivity Chip. (**A**) Cell-free DNA eluate from whole blood of patient 2, diluted 1:40; (**B**) cfDNA eluate from CTC-depl. blood of patient 2, diluted 1:40; (**C**) cfDNA eluate from whole blood of patient 3, diluted 1:5; (**D**) cfDNA eluate from CTC-depl. blood of patient 3, diluted 1:20.

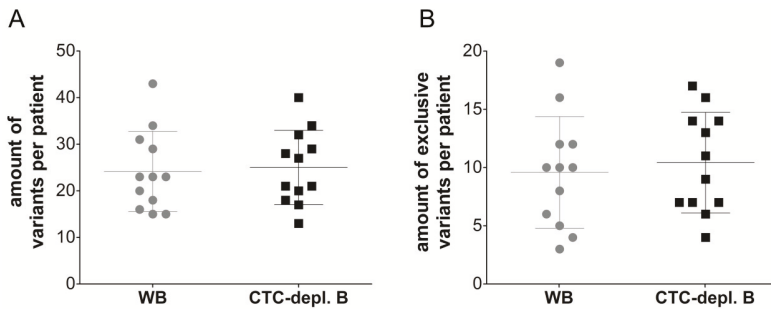
### 2.3. Cell-Free DNA Variants from Matched Whole Blood and Circulating Tumor Cell-Depleted Blood

Targeted deep sequencing was conducted for all cfDNA libraries >4 nM, resulting in removal of two matched cfDNA samples from one patient (Table S2, Supplementary Materials). Sequencing quality was guaranteed by exclusion of samples with <5 or >100 million read fragments, <400 unique molecular indice (UMI) coverage, and if <95% of the target region was covered with at least 5% of the mean UMI coverage, causing four samples and their matched samples to be rejected from analysis. The mean read count of all remaining samples from twelve patients was 12.41 million, while we found a mean of 3.5 reads per UMI and an average of 99.58% of the target region was covered with a least 5% of the mean UMI coverage (of 2760) (Table S2, Supplementary Materials).

In total, 415 variants were detected by UMI verification and, after filtering with the Ingenuity software, in all 24 cfDNA samples from 12 MBC patients, 175 variants were identical in the cfDNA samples from both plasma sources, namely WB and CTC-depl. B (Figure 3A). Furthermore, 115 and 125 variants were exclusively found in WB and CTC-depl. B, respectively. Thus, the agreement was 70% with a  $\kappa$ -value of 0.355 (Figure 3B). Corresponding to the large overlap of identical variants found in both plasma sources depicted in the Venn diagram (Figure 3A), the number of detected variants per patient exhibited no significant difference between the two matched cfDNA sources (Figure 4A).

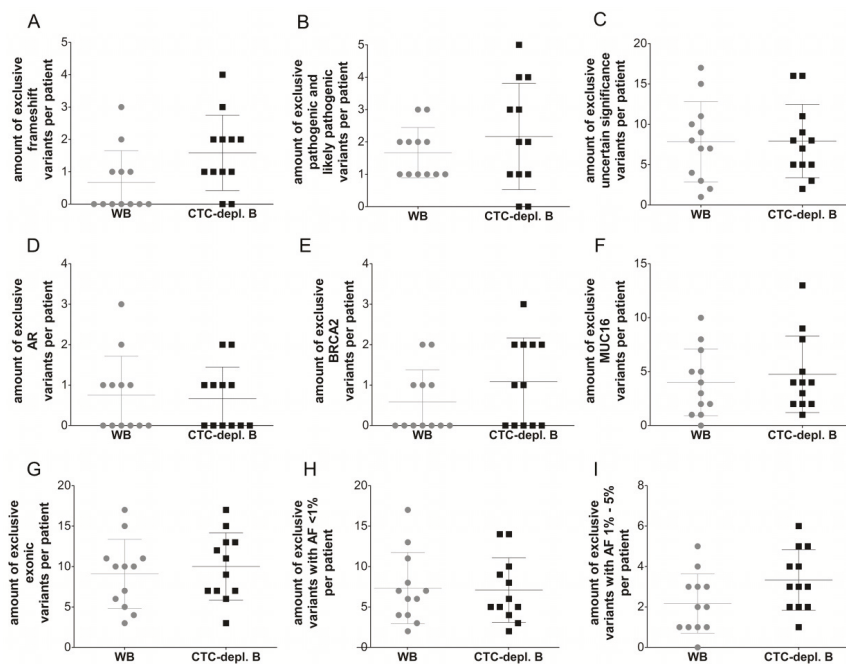


**Figure 3.** Venn diagram (A) and cross table (B) of matched cfDNA variants isolated from whole blood (light gray) and CTC-depleted blood (dark gray). Variants in all exonic regions of 17 genes were examined in 12 metastatic breast cancer (MBC) patients with hormone receptor positive/human epidermal growth factor receptor 2 negative (HR+/HER2-) primary tumor. Depletion of CTCs was conducted using AdnaTest EMT-2/StemCell Select. Variant calling by verification of unique molecular indices and filter of the Ingenuity Variant Analysis were described previously [25]. The proportional Venn diagram was computed using the tool BioVenn [26] and displays a great overlap of identical variants found in both cfDNA sources.



**Figure 4.** Boxplots describing the number of (A) detected cfDNA variants, and (B) the number of exclusively detected cfDNA variants per patient in whole blood (gray) and CTC-depleted blood (black, using AdnaTest EMT-2/StemCell Select). Means and standard deviations are also displayed, and the Wilcoxon signed-rank test indicated no significant difference between both cfDNA sources.

Additionally, the number of exclusively detected variants per patient in only one cfDNA source was also not significantly different (Figure 4B). Despite the similar quantity of variants found in only one of the two cfDNA sources, the characteristics of these variants might be different. Therefore, the number of variants per patient with the most common characteristics in the categories: translation impact, classification, gene, gene region, and allele frequency (AF) was compared in matched samples (Figure 5). The average number of exclusive frameshift variants, *BRCA2* variants, or variants with an allele frequency between 1% and 5% was slightly higher in the cfDNA samples isolated from CTC-depl. blood, but the Wilcoxon signed-rank test indicated no significant difference between the number of exclusive variants with specific characteristics (frameshift, pathogenic and likely pathogenic, uncertain significance, *AR*, *BRCA2*, *MUC16*, exonic, AF <1%, AF 1–5%) isolated from WB compared to variants isolated from CTC-depl. B. Therefore, we concluded that cfDNA variants from matched WB and CTC-depl. B harbor no differences.

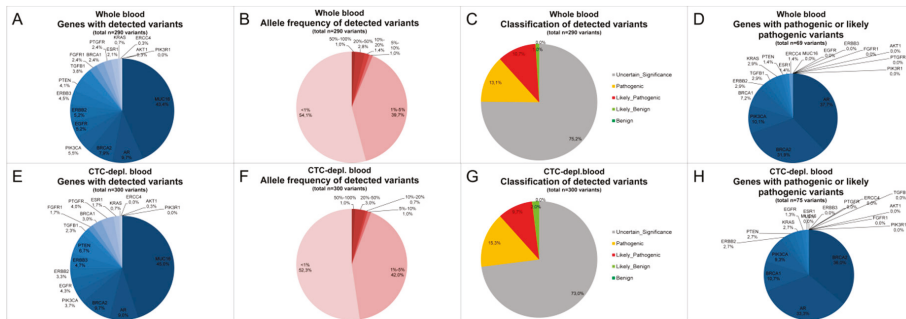


**Figure 5.** Boxplots describing the number of exclusively detected cfDNA variants per patient with specific characteristics in whole blood (gray) and CTC-depleted blood (black, using AdnaTest EMT-2/StemCell Select). The averages and standard deviations are indicated. Depicted characteristics are the major parameters of the categories: translation impact (frameshift (A)), classification (pathogenic and likely pathogenic (B), uncertain significance (C)), gene (*AR* (D), *BRCA2* (E), and *MUC16* (F)), gene region (exonic (G)), and allele frequency (<1% (H), 1–5% (I)). The Wilcoxon signed-rank test indicated no significant difference between the 12 matched cfDNA sources regarding any depicted characteristic.

#### 2.4. Clinically Relevant Characteristics of Cell-Free DNA Variants and Circulating Tumor Cell Expression

In the cohort of HR+/HER2– MBC patients, 50.0% of detected variants were located in the *MUC16* gene irrespective of the cfDNA source (Figure 6A,E). In total, 21.7%/22.5% of all variants isolated from WB/CTC-depl. B were located in the *AR* gene, and the third most commonly altered gene found was *BRCA2* (Figure 6A,E). Furthermore, 54.1%/52.3% of all variants had an AF of <1%, and 39.7%/42.0% of all variants isolated from WB/CTC-depl. B appeared with an AF of between 1% and 5% (Figure 6B,F). Moreover, 75.2%/73.0% of all variants were of uncertain significance, but 23.8%/25%

of the variants isolated from WB/CTC-depl. B were already described to be either pathogenic or likely pathogenic (Figure 6C,G). Around 30% of these 69/75 pathogenic or likely pathogenic variants in WB/CTC-depl. B were located in the *AR* or *BRCA2* gene, followed by variants in *BRCA1* (Figure 6D,H). In summary, the distribution of cfDNA variants was similar in samples isolated from WB and CTC-depl. B with most variants having a low AF, being mostly of uncertain significance and being located in the *MUC16*, *AR*, or *BRCA2* gene.



**Figure 6.** Distribution of called cfDNA variants isolated from whole blood (A–D) and CTC-depleted blood (E–H) of HR+/HER2– MBC patients ( $n = 12$ ) according to their gene location (A,D,E,H), allele frequency (B,F), and classification (C,G). (A,E) Distribution of (exclusive (D,H)) variants according to their gene location. Most variants were found in the *MUC16* gene, while pathogenic or likely pathogenic variants were mostly located in the *AR* or *BRCA2* gene. (B,F) Allele frequency of all variants; 90% of all variants showed AFs <5%. (C,G) Classification of variants according to their known impact (benign, likely benign, uncertain significance, likely pathogenic and pathogenic) done by IVA. Nearly 25% of all variants are known to be likely pathogenic or pathogenic. No significant different distribution was detected for cfDNA variants from matched whole blood and CTC-depl. blood.

To confirm that CTC analysis is feasible in addition to cfDNA analysis from the same blood sample, overexpression was examined by quantitative PCR (qPCR) in lysates of pooled CTCs. *mTOR* was the most commonly overexpressed transcript in CTCs of the studied cohort (Figure S1, Supplementary Materials). *AKT2* was also commonly overexpressed with a frequency of 88% in the cohort. *ERBB2*, *ERBB3*, *ERCC1*, *AURKA*, and *SRC* transcripts were overexpressed in the CTCs of more than 50% of all patients (Figure S1, Supplementary Materials).

### 3. Discussion

We here demonstrate that direct comparison of cfDNA variants isolated from matched WB and CTC-depl. B revealed no significant differences. The cfDNA concentration and size distribution, as well as the number of detected variants, were similar for both cfDNA sources. Even the characteristics of the exclusively detected cfDNA variants in either WB or CTC-depl. B showed no significant differences.

#### 3.1. Cell-Free DNA Sequencing from Circulating Tumor Cell-Depleted Blood Favored in the Future

The study was conducted to question the hypothesis that differences between WB and CTC-depl. B during cfDNA analysis occur, which were suspected to be caused by potential damage of CTCs and/or blood cells during CTC isolation, an influence of the immunomagnetic bead cocktail used for CTC enrichment, or an effect of additional 30-min incubation of WB at room temperature. However, since we found no differences in quantity and characteristics of the cfDNA variants isolated from WB versus CTC-depl. B, any systematic bias in sequencing cfDNA isolated from CTC-depl. B instead of WB can be excluded. Furthermore, we observed an unchanged sensitivity for cfDNA analysis using CTC-depl. B instead of WB. The blood supernatant remaining after positive immunomagnetic

selection of CTCs using the AdnaTest EMT-2/StemCell Select procedure can, therefore, be used as source for an additional liquid biopsy analyte. Isolation of CTCs and subsequent cfDNA isolation from CTC-depl. B using the QIAamp MinElute cfDNA Kit, followed by UMI-confirmed sequence analysis in a combined workflow, provide the advantage of cfDNA mutation analysis in concert with a CTC molecular profiling from exactly the same blood sample. However, in contrast to the original AdnaTest protocol, 5 mL of WB turned out to be not enough to obtain a CTC-depl. B volume sufficient for the subsequent cfDNA analysis, because targeted PCR-based deep sequencing of cfDNA requires a high input amount of cfDNA. Consequently, the CTC isolation process was conducted in duplicate from  $2 \times 5$  mL of WB to finally obtain minimally 4 mL of combined plasma from CTC-depl. B for cfDNA isolation with a yield  $>30$  ng. Thus, we describe a workflow to isolate the two liquid biopsy analytes cfDNA and CTCs from a minimal blood volume of only 10 mL, resulting in reduction of the burden of blood-draw volume for the patient, while simultaneously empowering a comprehensive liquid biopsy analysis.

### 3.2. Additive Value of Cell-Free DNA Mutational and Circulating Tumor Cell Transcriptional Analyses

A few studies comparing cfDNA and CTC data exist. However, in contrast to our present approach, for these studies, the blood volume needed for both analyses was additive and, thus, an additional burden for patients. Moreover, most of the isolation protocols required different preservative blood tubes for cfDNA and/or CTC storage. It was examined in BC patients that both total cfDNA level and CTC count were correlated with OS [17], that cfDNA integrity was correlated with CTC presence [20], that *SOX17* promoter methylation and *ESR1* methylation were highly concordant in cfDNA and CTCs [19,23], and that cfDNA and CTCs showed overlapping mutation profiles [17]. Using cfDNA variant analysis and CTC expression analysis of the same 10 mL of blood material, we here described the high prevalence of pathogenic or likely pathogenic *AR* variants (33.3%), and 38% of all 12 patients displayed an *AR* overexpression signal in CTCs. We conclude that *AR* might be of importance in HR+/HER2– MBC patients, both on a mutational and transcriptional level, as previously described by us in both cfDNA and CTCs in larger HR+/HER2– MBC cohorts [12,27]. Moreover, this gene is of relevance, because targeted treatment against *AR* is available; however, at the moment, it is only approved for other indications [28]. Furthermore, *AR* was described as a potential target in triple-negative BC patients [29,30].

Despite the similar results and consequences that can be drawn from cfDNA and CTC analysis as described above, cfDNA and CTCs also exhibit additive value. We here showed the high prevalence of *ERBB3* (70%), *ERBB2* (55%), and *PIK3CA* (27%) transcript overexpression in CTCs, whereas the prevalence of cfDNA variants isolated from matched CTC-depl. B and located in *ERBB3*, *ERBB2*, and *PIK3CA* genes was low (*ERBB3* 1.7%, *ERBB2* 2.2%, and *PIK3CA* 4.8%). This further exemplifies the additive value of CTC expression in combination with the cfDNA analysis. The protein expression of HER2 (encoded by *ERBB2*) is routinely used as a predictive marker for targeted therapy in BC [31]; therefore, the frequent overexpression of *ERBB2* transcripts in CTCs of patients with HER2- primary tumors, in line with previous results [12,32], might be relevant for treatment management in the future. Compared to cfDNA, CTCs provide a unique opportunity to study DNA, RNA, and proteins, even on the single-cell level, while also providing an indicator for active metastasis [33]. In particular, the expression analysis in CTCs was shown to be relevant for prognosis [34–36], prediction [37–39], and monitoring [11,12].

In contrast to CTC counts, cfDNA in MBC patients exhibited a greater correlation with tumor burden, cfDNA was more frequently found, cfDNA showed a greater dynamic range, and cfDNA described the earliest indication of response to chemotherapies [7]. Moreover, it was shown in BC patients that sequencing of cfDNA revealed more mutations than sequencing of CTCs [17], *ESR1* variant detection was more sensitive in cfDNA compared to CTCs [40], and cfDNA was correlated in a great extent with PFS than CTC counts [18]. Here, the existence of 1.3/10.7% pathogenic or likely pathogenic *EGFR/BRCA1* cfDNA variants accompanied by 0/25% overexpression frequency of

matched transcripts in CTCs also highlights the value of cfDNA in addition to CTC profiling. *EGFR* variant detection in cfDNA was the first liquid biopsy test to be approved by the USA Food and Drug Administration, as a companion diagnostic in non-small-cell lung cancer patients [41].

In multiple myeloma, it was shown that sequencing of both cfDNA and CTCs uncovered more mutations than in either analyte on its own, as in some cases a subclone was only detected in one analyte [13]. Thus, not only does the combined analysis of cfDNA variants and CTC transcriptional profiles provide additive information, but the mutational analysis of CTCs and cfDNA—from now on feasible using the same 10 mL of blood—is also assumed to be complementary rather than competitive [14].

## 4. Materials and Methods

### 4.1. Patient Population Characteristics and Eligibility Criteria

The study was conducted at the Department of Gynecology and Obstetrics, in collaboration with the Department of Medical Oncology (for specimen recruitment), both at the University Hospital Essen, Germany, and in collaboration with QIAGEN GmbH, Hilden, Germany (for library preparation and sequencing analysis). In accordance with the Declaration of Helsinki, written informed consent was obtained from all participants at enrolment, and specimens were collected using protocols approved in 2012 by the institutional review board that consists of medical doctors, nurses, psychologists, welfare workers, and a priest (12-5265-BO). In total, cfDNA from 17 MBC patients was studied between October 2015 and June 2018. All participants were  $\geq 18$  years, had Eastern Cooperative Oncology Group (ECOG) scores for performance status of 0–2, no severe, uncontrolled co-morbidities or medical conditions, and no second malignancies. Prior neoadjuvant and adjuvant treatment, radiation, all kinds of surgical intervention, or any other treatment of BC was permitted. Patients had estrogen (ER) and/or progesterone (PR) receptor-positive primary tumors (summarized as HR+) and no ERBB2 overamplification ( $n = 16$ ). Patients with ER-positive and/or PR-positive and HER2-negative metastases were also included if their ER, PR, and HER2 status in the primary tumor was unknown ( $n = 1$ ). Patient characteristics are listed in Table S1 (Supplementary Materials).

### 4.2. Sampling of Blood, Isolation of Circulating Tumor Cells and Processing of Plasma

Initially,  $2 \times 9$  mL of EDTA blood was collected in S-Monovettes (Sarstedt AG & Co, Nuembrecht, Germany), stored at  $4^\circ\text{C}$ , and processed within 4 h of blood draw. Five milliliters of whole blood were used in duplicate to isolate CTCs by positive immunomagnetic selection targeting EpCAM, EGFR, and HER2 (AdnaTest EMT-2/StemCell Select, QIAGEN, Hilden, Germany), as described in detail elsewhere [42]. The remaining matched whole blood not used for CTC isolation was centrifuged at  $3000 \times g$  for 8 min, and plasma was frozen at  $-80^\circ\text{C}$ . This plasma sample isolated straight from whole blood was abbreviated as WB. The CTC-depleted blood remaining after positive immunomagnetic selection, abbreviated as CTC-depl. B, was centrifuged at  $3000 \times g$  for 8 min, and plasma was frozen at  $-80^\circ\text{C}$ .

### 4.3. Isolation of Cell-Free DNA

Matched plasma samples from WB and CTC-depl. B were thawed, centrifuged at  $16,000 \times g$  for 10 min at  $4^\circ\text{C}$ , and passed through a  $0.8\text{-}\mu\text{m}$  pore size syringe filter (Sartorius, Goettingen, Germany). Cell-free DNA was isolated from 2.8–6.0 mL (mean 4.4 mL; maximized plasma volume available) plasma by affinity-based binding to magnetic beads according to the manufacturer's instructions (QIAamp MinElute ccfDNA Kit, QIAGEN) and as described previously [25]. The same volume of plasma was used for isolation of matched cfDNA from WB and CTC-depl. B of the same patient. Cell-free DNA was eluted in 22  $\mu\text{L}$  of ultraclean water and stored at  $-20^\circ\text{C}$ .



#### 4.4. Cell-Free DNA Quantification

Diluted cfDNA (1:5 to 1:100) was applied to an Agilent Chip High Sensitivity DNA (Santa Clara, CA, USA). Concentrations of fragments with a length between 100 and 700 bp were summed using the 2100 expert software B02.08 (Agilent) to calculate the cfDNA yield.

#### 4.5. Library Construction

The library was constructed with a customized QIAseq Targeted DNA Panel Kit (QIAGEN), as described in detail previously [25,27]. The input amount preferred for library preparation was in the range of 30–60 ng, but cfDNA samples with lower input were also included in the library preparation. The same cfDNA input amount of matched cfDNA samples from WB and CTC-depl. B was used for the library preparation, as listed in Table S2 (Supplementary Materials). Thus, the mean cfDNA input used across the cohort was 54.47 ng. We applied a 20  $\mu$ L input volume in accordance with the protocol previously described [25]. Briefly, end-repair and A-addition was performed while the enzymatic fragmentation was inhibited. Subsequently, barcoded adapters including the UMI and sample-specific indices were ligated to the fragments. DNA was purified and free adapters were depleted by magnetic beads. The targeted enrichment was performed with a customized QIAGEN QIAseq Targeted DNA Panel primer designed to amplify all coding regions of the following genes: *AKT1*, *AR*, *BRCA1*, *BRCA2*, *EGFR*, *ERBB2*, *ERBB3*, *ERCC4*, *ESR1*, *KRAS*, *FGFR1*, *MUC16*, *PIK3CA*, *PIK3R1*, *PTEN*, *PTGFR*, and *TGFB1*. The universal PCR amplification was followed by a magnetic bead clean-up, and the final targeted enriched cfDNA library was eluted.

#### 4.6. Sequencing

Libraries were quantified as published [25] by qPCR and the quality was checked using an Agilent Chip High Sensitivity DNA (Santa Clara). Libraries were diluted to 4 nM, and libraries with a lower yield (and matched libraries from the same patient;  $n = 1$ ) were excluded. All pooled libraries were analyzed by paired-end sequencing on the Illumina NextSeq Sequencer with a NextSeq 550 System High-Output Kit,  $2 \times 150$ -bp reads using a custom sequencing primer (QIAseq A Read1 Primer, QIAGEN).

#### 4.7. Data Analysis/Bioinformatical Analysis

Data were initially analyzed using the QIAGEN GeneGlobe Data Analysis Center. Sufficient sequencing quality of all samples was guaranteed by exclusion of libraries with less than five million read fragments ( $n = 1$ ), a UMI coverage lower than 400 ( $n = 1$ ), and if less than 95% of the target region was covered with at least 5% of the mean UMI coverage ( $n = 1$ ) [25,27]. Furthermore, libraries with >100 million read fragments were excluded to remove highly overrepresented libraries ( $n = 1$ ). The matched libraries of the excluded ones were not analyzed to also guarantee comparison of only matched samples. The input amount, library yield, and sequencing quality parameter of each sample are summarized in Table S2 (Supplementary Materials). The QIAGEN Biomedical Genomics Workbench and the Ingenuity Variant Analysis plugin (IVA; QIAGEN) were used for further annotation, scoring, filtering (described previously [25]), and interpretation of variants detected in the UMI-based analysis. In detail, a consensus sequence was built from all fragments with the same UMI to exclude potential artefacts.

The IVA used five different filters for exclusion of called variants. The confidence filter excluded all variants with a call quality below 20. Moreover, the confidence filter only kept variants that were not located in the top 5% of the most exonically variable 100-base windows in healthy public genomes. Variants with a prevalence of >0.5% in the normal population (reference databases: (1) Allele Frequency Community (gnomAD&CGI), (2) 1000 Genomes Project, (3) ExAC, and (4) NHLBI ESP exomes) were excluded, unless the variant was already known to be a pathogenic common variant, to identify rare variants potentially associated with the evaluated condition of the tested cohort (common

variant filter). The genetic analysis filter only kept variants that were associated with gain of function or with the following inheritance patterns: homozygous, compound heterozygous, haploinsufficient, hemizygous, het-ambiguous, or heterozygous. Only variants located no more than 20 bases from the intron were included, as well as those described to be pathogenic and/or likely pathogenic from the American College of Medical Genetics and Genomics, or variants that are loss-of-function-associated, which induce frameshift, in-frame indel, start/stop changes, missense, intronic two-bp splice-site loss, or any splice-alteration predicted by MaxEntScan (predicted deleterious filter). The cancer driver variant filter kept only variants that were found in (1) cancer-associated mouse knockout phenotypes, (2) cancer-associated cellular processes with any directionality, (3) cancer-associated pathways with any directionality, (4) cancer therapeutic targets, (5) published cancer literature variants and gene level findings, (6) known or predicted cancer subnetwork regulatory sites, (7) COSMIC at a frequency greater than or equal to 0.01%, and (8) TCGA at a frequency greater than or equal to 0.01%.

Original raw sequencing data were uploaded as two fastq files plus MD5 checksum per sample, and are available at the European Nucleotide Archive with the study accession number PRJEB30449.

#### 4.8. Messenger RNA Isolation and Quantitative PCR

Messenger RNA was isolated from the CTC lysates by oligo(dT)<sub>25</sub>-coated magnetic beads and was reverse-transcribed (Adna-Test EMT-2/StemCell Detect, QIAGEN; [42]). The AdnaTest TNBC Panel prototype (QIAGEN), consisting of multimarker real-time (RT)-qPCR assays, was applied to the complementary DNA isolated from the CTCs for expression profiling of *AKT2*, *ALK*, *AR*, *AURKA*, *BRCA1*, *EGFR*, *ERCC1*, *ERBB2*, *ERBB3*, *KIT*, *KRT5*, *MET*, *MTOR*, *NOTCH1*, *PARP1*, *PIK3CA*, *SRC*, and *GAPDH*, in relation to the leukocyte-specific transcript *CD45* and healthy donor controls. Experimental set-up and data evaluation were described in detail previously [12].

#### 4.9. Statistical analysis

Statistical analysis was performed using SPSS, version 11.5 (IBM, Armonk, NY, USA). The Wilcoxon signed-rank test was used to assess whether the matched samples differed. Diagrams were computed with GraphPad PRISM (GraphPad Software Inc., San Diego, CA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Box plots display individual values for each patient, as well as the mean and standard deviation. The Venn diagram was produced with the online tool BioVenn [26]. The kappa-value was calculated using the GraphPad QuickCalcs website [43].

## 5. Conclusions

In summary, cfDNA mutational and CTC transcriptional analyses can supplement each other. A comprehensive liquid biopsy, therefore, increases the chance for identification of actionable targets to direct therapy strategies. Thus, the isolation of both CTCs and cfDNA, in an “all from one tube” format without bias due to the initial CTC isolation process, as demonstrated in this research study, might be helpful in paving a way for individual treatment decision-making.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/11/2/238/s1>: Figure S1: Overexpression signal frequency in pooled CTCs all of blood samples ( $n = 12$ ) subsequently called CTC-depleted blood and used for cfDNA isolation; Table S1: Patient characteristics; Table S2: Sequencing qualities.

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Article

# Lactate Dehydrogenase (LDH) Response to First-Line Treatment Predicts Survival in Metastatic Breast Cancer: First Clues for a Cost-Effective and Dynamic Biomarker

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**Abstract:** Background: Elevated plasmatic lactate dehydrogenase (LDH) levels are associated with worse prognosis in various malignancies, including metastatic breast cancer (MBC). Nevertheless, no data are available on the prognostic role of LDH as a dynamic biomarker during first-line treatment in unselected MBC. Methods: We reviewed data of 392 women with MBC to evaluate the association between LDH variation after 12 weeks of first-line treatment and survival. The prognostic impact was tested by multivariate Cox regression analysis. Results: Plasmatic LDH was confirmed as an independent prognostic factor in MBC. Patients who maintained elevated LDH levels after 12 weeks of first-line treatment experienced worse progression-free survival (PFS, HR 2.88, 95% CI: 1.40–5.89,  $p = 0.0038$ ) and overall survival (OS, HR 2.61, 95% CI 1.16–5.86,  $p = 0.02$ ) compared to patients with stable normal LDH levels, even after adjustment for other prognostic factors. Notably, LDH low-to-high variation emerged as an unfavorable prognostic factor for PFS (HR 3.96, 95% CI 2.00–7.82,  $p = 0.0001$ ). Conclusions: Plasmatic LDH and its variation during first-line treatment predict PFS and OS in MBC, providing independent prognostic information. It would be worthwhile to prospectively evaluate the association between LDH variation and therapeutic benefit in MBC, and explore how it may affect treatment strategies.

**Keywords:** metastatic breast cancer; lactate dehydrogenase; serum biomarker; LDH; monitoring metastatic breast cancer

## 1. Introduction

Breast cancer (BC) is the most common cancer among women and the second leading cause of cancer-related death [1]. About 6% of all breast tumors present with distant metastases at diagnosis, and 30% of patients with early BC will experience local or distant recurrence [2]. BC is a heterogeneous disease, including distinct subgroups with different prognosis based on histological and molecular features [3]. In clinical practice, the expression of the estrogen receptor (ER), progesterone receptor

(PR), and the human epidermal growth factor receptor 2 (HER2) identifies three main subgroups: Luminal or hormone receptor positive (HR-positive) BC, HER2-positive BC, and triple negative breast cancer (TNBC) [4]. Despite new treatments and improved standard of care, metastatic breast cancer (MBC) remains an incurable disease with a median survival of about 34 months, even if it varies significantly among and within the subgroups [5]. Therefore, it is essential to identify tumor- and patient-related factors able to predict aggressive biological behavior and treatment resistance. Recently, several studies evaluated novel circulating biomarkers in BC, including inflammatory factors [6], exosomes [7], circulating tumor DNA (ctDNA) [8], and circulating tumor cells (CTC) [9]. However, even routinely used biomarkers (e.g., the neutrophil-to-lymphocyte ratio [10], lactate dehydrogenase (LDH) [11], alkaline phosphatase (ALP) [12]) provide additional information on tumor biology and should be further evaluated for their prognostic relevance.

LDH is a ubiquitous enzyme that plays a central role in anaerobic glycolysis, as it catalyzes the reversible conversion of pyruvate into lactate [13]. LDH comprises a family of six tetrameric isoenzymes [14,15] with a tissue-specific expression regulated by both physiological and pathological conditions. The *LDHA* gene expression is upregulated in several types of cancers, especially in rapidly growing tumors, to maintain glycolysis as an alternative source of energy during hypoxic stress and subsequent high LDH level in cytoplasmic compartment. Notably, different extracellular factors, such as hormones, growth factors, and cytokines can regulate LDH expression by receptor-dependent and -independent intracellular signaling pathways (e.g., cAMP Response Element-Binding protein (CREB), Hypoxia-Inducible Factor-1 (HIF-1), and c-Myc) [15]. Beyond its role in regulating cellular metabolism, LDH is a well-known marker of tissue damage. Many pathological conditions, including cancer, present with LDH elevation due to acute cell death or necrosis. Moreover, high plasmatic LDH levels influence tumor progression and metastatic spread with a negative impact on outcome in various cancer types [16–25].

The prognostic role of plasmatic LDH levels has been investigated in BC as well. The first piece of evidence dates back to the late 1990s and early 2000s when three extensive studies found that elevated plasmatic LDH levels were associated with poor outcome in MBC patients [26–28]. High plasmatic LDH levels were also proven to be significantly associated with increased risk of disease recurrence and death [12,29]. Notably, a recent meta-analysis confirmed these findings in both MBC and early BC [11].

Nevertheless, no data are available on the prognostic role of LDH dynamic response to first-line treatment in unselected MBC patients. Thus, we conducted an exploratory study to identify the prognostic impact of plasmatic LDH variation after 12 weeks of first-line treatment on both progression-free survival (PFS) and overall survival (OS) in MBC.

## 2. Results

### 2.1. Patient's Characteristics

A consecutive series of 392 women with MBC were included in the analysis, 219 with a plasmatic LDH evaluation at baseline. The median age was 62 years (range 29–88), with 42.9% of patients older than 65 years and 10.7% younger than 45 years. Invasive ductal carcinoma was the most common histology (80.4% of cases), and post-menopausal women accounted for 59.4% of patients. Approximately 60.5% of patients had HR-positive tumors (11.2% were luminal A, 38.3% luminal B, and 11.0% luminal HER2-positive; see Section 4.2. for classification details), 8.7% had HR-negative/HER2-positive disease, and 9.4% TNBC. At MBC diagnosis, nearly half of the patients presented with a single metastatic site, and about 20% had three or more localizations. Bone metastases were detected in half of the cases (20% of patients had a bone-only disease), while patients with liver, lung, or central nervous system localizations (CNS) were about 25%, 28%, and 6.4%, respectively. Overall, nearly 60% of patients received chemotherapy as first-line treatment, and the remaining 40% received hormonal therapy. Additional baseline clinical and pathologic characteristics of patients are listed in Table 1.

**Table 1.** Baseline patients' clinical and pathologic characteristics.

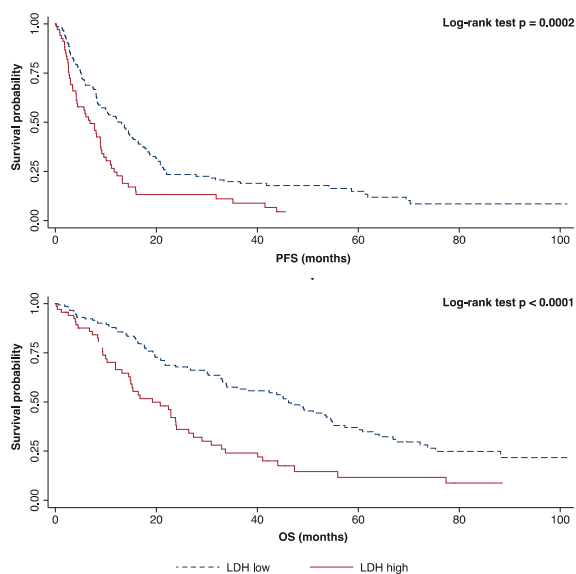
Characteristics		Number of Patients (Total = 392)	%
Age	<45 years	42	10.71
	45–65 years	182	46.43
	>65 years	168	42.86
Menopausal state	Pre-menopausal	114	29.08
	Post-menopausal	233	59.44
	Unknown	45	11.48
Histotype	Ductal	315	80.36
	Lobular	59	15.05
	Other	12	3.06
	Unknown	6	1.53
Profile	Luminal A	44	11.22
	Luminal B	150	38.27
	Luminal HER2	43	10.97
	HER2-positive	34	8.67
	Triple negative	37	9.44
ECOG PS	Unknown	84	21.43
	0	201	51.28
	1	150	38.26
	≥2	34	8.67
Number of metastatic sites	Unknown	7	1.79
	1	212	54.08
	2	104	26.53
Site of metastases *	≥3	76	19.39
	Bone	199	50.77
	Bone only	79	20.15
	Liver	99	25.26
	CNS	25	6.38
Firs-line treatment	Lung	110	28.06
	Lymph nodes	133	33.93
	Chemotherapy	231	58.93
	Hormonal therapy	161	41.07
	Baseline LDH level	High <sup>1</sup>	69
Normal		150	38.27
Unknown		173	44.13
Baseline ALP level	High <sup>2</sup>	124	31.63
	Normal	245	62.50
	Unknown	23	5.87

Legend: CNS, Central Nervous System; LDH, Lactate dehydrogenase; ALP, alkaline phosphatase; ECOG PS, Eastern Cooperative Oncology Group Performance Status. <sup>1</sup> LDH > 480 IU/L; <sup>2</sup> ALP > 104 IU/L; \* Patients may present more than one metastatic site.

## 2.2. Prognostic Role of Pre-Treatment Plasmatic LDH

After a median follow-up of 52.77 months, median OS was 30.87 months (25–75th percentile: 13.50–62.80), and median PFS was 9.21 months (25–75th percentile: 3.95–20.70). At baseline, 31.5% of evaluable patients (69/219) had elevated pre-treatment LDH levels according to the centralized laboratory cut-off (>480 IU/L). Through univariate analyses, baseline elevated plasmatic LDH emerged as an unfavorable prognostic factor in terms of PFS and OS. More specifically, patients with baseline elevated LDH experienced shorter median PFS (6.87 vs. 13.12 months, HR 1.81, 95% CI: 1.31–2.51,  $p = 0.0003$ ) and OS (19.23 vs. 46.19 months, HR 2.23, 95% CI: 1.55–3.19,  $p < 0.0001$ ) compared to patients with normal LDH (Figure 1). The prognostic role of LDH plasma levels was also confirmed when evaluated as a continuous variable for both PFS ( $p = 0.0002$ ) and OS ( $p < 0.0001$ ).





**Figure 1.** Kaplan–Meier curves for progression-free survival (PFS) and overall survival (OS) according to baseline lactate dehydrogenase (LDH).

These findings were confirmed for both PFS (HR 1.51, 95% CI: 1.02–2.26,  $p = 0.039$ ) and OS (HR 1.64, 95% CI: 1.05–2.55,  $p = 0.027$ ) after multivariate adjustment for molecular profiles, Eastern Cooperative Oncology Group Performance Status (ECOG PS), baseline ALP level, number of metastatic sites, central nervous system (CNS), and liver and bone localizations (Tables 2 and 3).

**Table 2.** Baseline prognostic factors for PFS according to univariate and multivariate Cox model.

Covariates		Number of Patients	Univariate Analysis(HR, 95% CI)	$p$	Multivariate Analysis(HR, 95% CI)	$p$
Age	<45 years	42	0.80 (0.54–1.17)	0.25		
	45–65 years	182	Ref.	-		
	>65 years	168	0.95 (0.75–1.20)	0.68		
Profile	Luminal A	44	Ref.	-	Ref.	-
	Luminal B	150	1.27 (0.87–1.87)	0.20	1.10 (0.66–1.84)	0.68
	Luminal HER2	43	0.73 (0.44–1.19)	0.21	0.58 (0.31–1.10)	0.09
	HER2-positive	34	1.17 (0.71–1.92)	0.52	0.92 (0.44–1.92)	0.84
	Triple negative	37	3.19 (1.96–5.17)	<0.0001	2.81 (1.44–5.48)	0.002
ECOG PS	0	201	Ref.	-	Ref.	-
	1	150	1.25 (0.98–1.58)	0.06	1.35 (0.90–2.02)	0.13
	≥2	34	1.70 (1.12–2.59)	0.01	2.45 (1.18–5.07)	0.01
Number of metastatic sites	1	212	Ref.	-	Ref.	-
	2	104	1.35 (1.04–1.75)	0.02	1.51 (0.97–2.35)	0.06
	≥3	76	0.97 (0.71–1.34)	0.89	0.61 (0.35–1.04)	0.07
Site of metastases *	Bone	199	0.93 (0.74–1.16)	0.54	1.07 (0.71–1.61)	0.73
	Liver	99	1.14 (0.88–1.47)	0.29	0.92 (0.58–1.47)	0.75
	CNS	25	1.38 (0.86–2.20)	0.17		
	Lung	110	0.90 (0.70–1.16)	0.44		
Baseline LDH level	High <sup>1</sup>	69	1.81 (1.31–2.51)	0.0003	1.51 (1.02–2.26)	0.039
	Normal	150	Ref.	-	Ref.	-
Baseline ALP level	High <sup>2</sup>	124	1.45 (1.14–1.85)	0.002	1.11 (0.73–1.66)	0.61
	Normal	245	Ref.	-	Ref.	-

Legend: CNS, Central Nervous System; LDH, Lactate dehydrogenase; ALP, alkaline phosphatase; ECOG PS, Eastern Cooperative Oncology Group Performance Status; Ref., Reference. <sup>1</sup> LDH cut-off: 480 IU/L; <sup>2</sup> ALP cut-off: 104 IU/L; \* Patients may present more than one metastatic site.

**Table 3.** Baseline prognostic factors for OS according to univariate and multivariate Cox model.

Covariates		Number of Patients	Univariate Analysis(HR, 95% CI)	<i>p</i>	Multivariate Analysis(HR, 95% CI)	<i>p</i>
Age	<45 years	42	0.82 (0.52–1.29)	0.41		
	45–65 years	182	Ref.	-		
	>65 years	168	1.16 (0.89–1.53)	0.25		
Profile	Luminal A	44	Ref.	-	Ref.	-
	Luminal B	150	1.63 (1.01–2.62)	0.04	2.26 (1.16–4.38)	0.01
	Luminal HER2	43	1.14 (0.63–2.03)	0.65	1.73 (0.81–3.70)	0.15
	HER2-positive	34	1.61 (0.88–2.96)	0.12	1.24 (0.49–3.15)	0.64
	Triple negative	37	4.31 (2.45–7.59)	<0.0001	7.19 (3.11–16.5)	<0.0001
ECOG PS	0	201	Ref.	-	Ref.	-
	1	150	1.92 (1.45–2.55)	<0.0001	1.88 (1.18–2.99)	0.007
	≥2	34	2.61 (1.72–3.97)	<0.0001	1.76 (0.84–3.70)	0.13
Number of metastatic sites	1	212	Ref.	-	Ref.	-
	2	104	1.40 (1.03–1.89)	0.02	2.04 (1.20–3.46)	0.008
	≥3	76	1.36 (0.95–1.94)	0.08	0.70 (0.35–1.41)	0.32
Site of metastases *	Bone	199	0.95 (0.73–1.23)	0.74	1.19 (0.73–1.96)	0.47
	Liver	99	1.33 (1.00–1.78)	0.046	0.58 (0.68–1.93)	0.58
	CNS	25	2.72 (1.68–4.42)	<0.0001	22.05 (4.38–110.94)	0.0002
	Lung	110	1.10 (0.82–1.47)	0.51		
Baseline LDH level	High <sup>1</sup>	69	2.22 (1.55–3.19)	<0.0001	1.64 (1.05–2.54)	0.027
	Normal	150	Ref.	-	Ref.	-
Baseline ALP level	High <sup>2</sup>	124	1.84 (1.40–2.41)	<0.0001	1.48 (0.94–2.31)	0.08
	Normal	245	Ref.	-	Ref.	-

Legend: CNS, Central Nervous System; LDH, Lactate dehydrogenase; ALP, alkaline phosphatase; ECOG PS, Eastern Cooperative Oncology Group Performance Status; Ref., Reference. <sup>1</sup> LDH cut-off: 480 IU/L; <sup>2</sup> ALP cut-off: 104 IU/L;

\* Patients may present more than one metastatic site.

The role of LDH as an adverse prognostic factor was consistent in all examined subgroups: Age, profile, number of metastatic sites, type of first-line treatment (hormonal therapy or chemotherapy), baseline ALP level, and liver and bone involvement (Figure 2). Aside from baseline LDH level, other independent prognostic factors for PFS were triple negative profile (HR 2.81, 95% CI: 1.44–5.48,  $p = 0.002$ ) and ECOG PS (2 vs. 0, HR 2.45, 95% CI: 1.18–5.07,  $p = 0.015$ ), while for OS, they were luminal B profile (HR 2.26, 95% CI: 1.16–4.38,  $p = 0.015$ ), triple negative profile (HR 7.19, 95% CI: 3.11–16.58,  $p < 0.0001$ ), ECOG PS (1 vs. 0, HR 1.88, 95% CI: 1.18–2.99,  $p = 0.007$ ), tumor burden (2 vs. 1 localizations, HR 2.04, 95% CI: 1.20–3.46,  $p = 0.008$ ), and CNS localizations (HR 22.05, 95% CI: 4.38–110.94,  $p = 0.002$ ). The complete Cox regression model is reported in Tables 2 and 3.

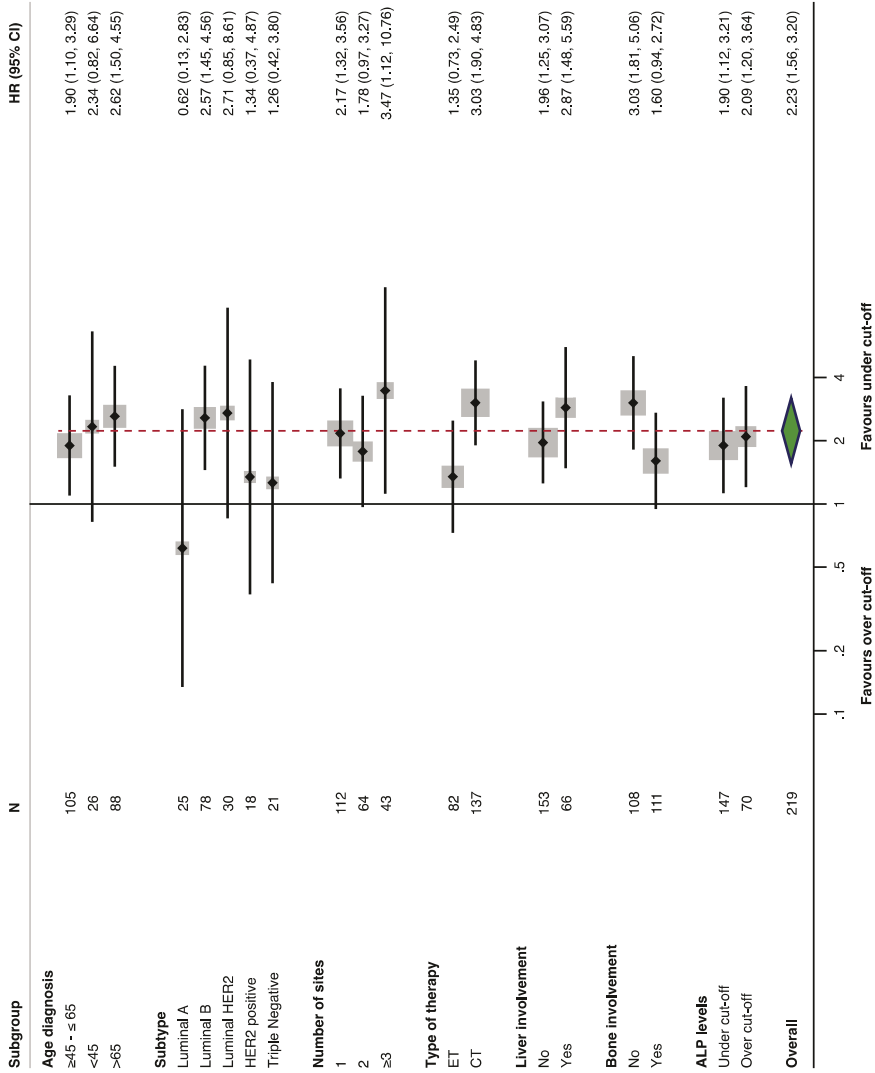


Figure 2. Subgroup analysis of OS in patients with baseline elevated LDH vs. normal LDH level.

2.3. Prognostic Role of Plasmatic LDH Response during First-Line Treatment.

LDH value after 12 weeks of first-line treatment was available in 126 patients (32%). Among them, 54.7% had stable low LDH levels, 15.0% had stable high levels, and in approximately 30% of cases, LDH levels changed over time across the upper normal limit (12% had a drop under the upper normal limit, while 18.2% had a rise over the upper normal limit).

According to plasmatic LDH variation, we were able to detect significant differences of both median PFS (stable low levels: 18.71 months, high-to-low levels: 10.92 months, low-to-high levels: 5.13 months, stable high levels: 4.27 months,  $p < 0.0001$ ) and median OS (stable low levels: 54.64 months, high-to-low levels: 30.87 months, low-to-high levels: 29.49 months, stable high levels: 14.83 months,  $p < 0.0001$ ) (Figure 3 and Table 4).

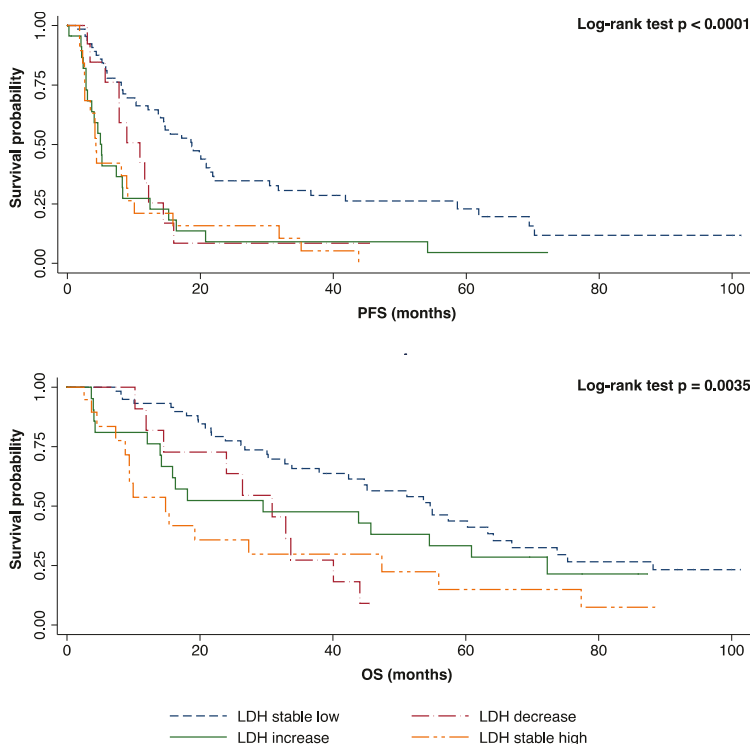


Figure 3. Kaplan–Meier curves for PFS and OS according to plasmatic LDH variation after 12 weeks of first-line treatment.

Table 4. OS and PFS according to plasmatic LDH variation after 12 weeks of first-line treatment.

LDH Variation <sup>1</sup>	Number of Patients (Total = 126)	%	Median PFS (25–75th Percentile)	Median OS (25–75th Percentile)
Stable low	69	54.76	18.71 (8.09–58.65)	54.64 (26.76–88.18)
High-to-low	15	11.91	10.92 (7.76–14.43)	30.87 (14.53–40.08)
Low-to-high	23	18.25	5.13 (2.79–12.43)	29.49 (14.01–72.26)
Stable high	19	15.08	4.27 (2.60–10.06)	14.83 (8.78–47.38)

Legend: LDH, Lactate dehydrogenase. <sup>1</sup> LDH cut-off: 480 IU/L.

The prognostic relevance of LDH response to first-line treatment was then assessed using a Cox regression multivariate model. Stable elevated LDH levels after 12 weeks of first-line treatment was confirmed as an independent negative prognostic factor for both PFS (HR 2.88, 95% CI: 1.40–5.89,  $p = 0.0038$ ) and OS (HR 2.61, 95% CI 1.16–5.86,  $p = 0.02$ ) after multivariate adjustment for molecular profile, ECOG PS, number of metastatic sites, CNS, liver, bone localizations, and plasmatic ALP variation at 12 weeks. Moreover, a rise in plasmatic LDH levels after 12 weeks of first-line treatment (low-to-high variation) also emerged by multivariate analysis as an independent negative prognostic factor for PFS (HR 3.96, 95% CI 2.00–7.82,  $p = 0.0001$ ) with a trend for worse OS (HR 2.02, 95% 0.89–4.56,  $p = 0.08$ ). The complete Cox regression model is reported in Table 5.

**Table 5.** LDH variation after 12 weeks of first-line treatment: Prognostic impact on PFS and OS according to multivariate Cox model.

Covariates		Number of Patients	Multivariate Analysis (HR, 95% CI) PFS	$p$	Multivariate Analysis (HR, 95% CI) OS	$p$
Profile	Luminal A	44	Ref.	-	Ref.	-
	Luminal B	150	0.88 (0.43–1.80)	0.73	1.39 (0.57–3.36)	0.46
	Luminal HER2	43	0.37 (0.17–0.81)	0.01	0.75 (0.30–1.86)	0.54
	HER2-positive	34	1.12 (0.41–3.03)	0.12	0.66 (0.18–2.40)	0.53
	Triple negative	37	2.90 (1.16–7.22)	0.02	7.81 (2.66–22.9)	0.0002
ECOG PS	0	201	Ref.	-	Ref.	-
	1	150	1.68 (0.90–3.14)	0.10	1.78 (0.84–3.79)	0.13
	≥2	34	4.19 (1.48–11.85)	0.006	2.29 (0.81–6.47)	0.11
Number of metastatic sites	1	212	Ref.	-	Ref.	-
	2	104	1.86 (0.92–3.73)	0.08	1.75 (0.79–3.88)	0.16
	≥3	76	0.71 (0.32–1.56)	0.40	0.67 (0.25–1.79)	0.43
Site of metastases *	Bone	199	1.46 (0.76–2.81)	0.25	2.35 (1.05–5.23)	0.036
	Liver	99	0.88 (0.44–1.76)	0.72	1.53 (0.71–3.31)	0.26
	CNS	25			1223.5 (42.5–35225.6)	<0.0001
ALP variation at 12 weeks <sup>1</sup>	Stable low	20262	Ref.	-	Ref.	-
	High-to-low	44	0.82 (0.35–1.87)	0.63	0.62 (0.25–1.52)	0.30
	Low-to-high	13	0.88 (0.22–3.42)	0.86	2.39 (0.57–10.0)	0.23
	Stable high	62	0.98 (0.45–2.12)	0.96	1.24 (0.53–2.88)	0.60
LDH variation at 12 weeks <sup>2</sup>	Stable low	69	Ref.	-	Ref.	-
	High-to-low	15	1.27 (0.50–3.23)	0.60	2.35 (0.82–6.77)	0.11
	Low-to-high	23	3.96 (2.00–7.82)	0.0001	2.02 (0.89–4.56)	0.08
	Stable high	19	2.88 (1.40–5.89)	0.003	2.61 (1.16–5.86)	0.02

Legend: CNS, Central Nervous System; LDH, Lactate dehydrogenase; ALP, alkaline phosphatase; Ref., Reference; ECOG PS, Eastern Cooperative Oncology Group Performance Status. <sup>1</sup> ALP cut-off: 104 IU/L; <sup>2</sup> LDH cut-off: 480 IU/L; \* Patients may present more than one metastatic site.

### 3. Discussion

Many studies reported elevated plasmatic LDH levels to be associated with poor outcomes in various tumors [30]. A recent meta-analysis, including 76 studies conducted in patients with several cancer types, confirmed that high LDH plasmatic levels were associated with shorter PFS and OS [31]. Although the prognostic role of LDH in cancer is well-established, the underlying biological mechanisms are still unclear, and some possible explanations have been hypothesized. Firstly, high LDH plasmatic concentrations sustain anaerobic metabolism during tumor growth and metastatic spread, supporting the energetic requirements in hypoxic conditions [32]. Secondly, LDH exerts an inflammatory action on tumor microenvironment, activating interleukin (IL)-23 and IL-17 and modulating the activity of arginase I. It inhibits CD8+ T lymphocytes and natural killer (NK) activation, allowing cancer cells to evade immune response [33]. Moreover, high LDH levels promote tumor angiogenesis, cell migration, and metastatization by inhibiting the degradation of HIF-1 alpha and increasing the production of vascular endothelial growth factor (VEGF) [34]. Thirdly, preliminary evidence suggests that increased *LDHA* expression and lactate overproduction might also play a role in drug resistance [35].

The present study investigated the prognostic impact of plasmatic LDH levels on survival outcomes in MBC patients at first-line treatment.

Approximately 31% of evaluated patients had high baseline LDH levels and about 32% had an LDH variation during first-line treatment. In particular, 15% of patients had a stable high LDH and 18% had a low-to-high variation.

The results confirmed that elevated baseline LDH levels were independently associated with shorter PFS (6.87 vs. 13.12 months, adjusted HR 1.51, 95% CI: 1.02–2.26,  $p = 0.039$ ) and OS (19.23 vs. 46.19 months, adjusted HR 1.64, 95% CI: 1.05–2.55,  $p = 0.027$ ). These data were also confirmed when LDH plasma levels were evaluated as a continuous variable (PFS, OS), so our results were not dependent on the pre-specified cut-off for normal LDH plasmatic concentrations. To the best of our knowledge, this is the first study to demonstrate that LDH changes during first-line treatment significantly impact both PFS and OS in unselected MBC patients. Specifically, patients with elevated baseline plasmatic LDH who maintained high LDH levels after 12 weeks of first-line treatment experienced worse PFS and OS compared to patients with stable normal LDH levels, even after adjustment for other prognostic factors (HR 2.88, 95% CI: 1.40–5.89,  $p = 0.0038$  and HR 2.61, 95% CI 1.16–5.86,  $p = 0.02$  for OS and PFS, respectively). Interestingly, since elevated plasmatic LDH levels may also reflect the presence of high tumor burden, bone localizations, liver metastases, and ALP levels variations, it is noteworthy that their prognostic value was maintained after including these covariates in the multivariate Cox regression model.

Additionally, plasmatic LDH elevation during first-line treatment emerged as an independent prognostic factor for PFS (HR 3.96, 95% CI 2.00–7.82,  $p = 0.0001$ ) with a trend for OS (HR 2.02, 95% 0.89–4.56,  $p = 0.08$ ). In accordance with our findings, a recent study conducted in TNBC patients confirmed that LDH changes after two cycles of first-line chemotherapy correlate with objective response rate and PFS [36].

Therefore, LDH can predict survival in patients with MBC and provides independent and dynamic prognostic information during first-line treatment. Given our results, patients with stable high LDH levels or with LDH elevation during first-line therapy may be monitored more frequently for disease progression, as they might experience shorter PFS. Conversely, patients with stable normal LDH levels will experience prolonged PFS and OS. Nevertheless, since these findings are not prospectively validated, LDH variation must not be considered an indirect proof of tumor progression or response, even if it offers additional prognostic information.

In our study, LDH-A tissue expression was not tested. However, its relationship with plasmatic LDH may be useful to define whether LDH plasmatic elevation is primarily tumor-related or not, exploring the biological significance and the prognostic value of their concordance or discordance. According to previous studies, elevated tissue LDH-A expression is associated with elevated Ki-67, high proliferation rates, and CNS metastases in TNBC [37].

The main strength of our study is the identification of a dynamic, easy-to-use, inexpensive, and reproducible prognostic biomarker in patients with unselected MBC. However, this is a retrospective and single-center study. Thus, prospective and external validation is mandatory. Moreover, the LDH cut-off value for normality implemented in this study may differ in other centers; consequently, its reproducibility has to be confirmed. Lastly, we did not consider the potential interaction of several other non-neoplastic diseases (e.g., heart failure, anemia, hypothyroidism, autoimmune, and lung disorders), which might influence plasmatic LDH levels.

On the basis of these observations, it would be of great value to prospectively evaluate the potential correlation between LDH variation and response to treatment in MBC, and explore the prognostic role of this long-standing biomarker in the modern era of immunotherapy and targeted therapy.

## 4. Materials and Methods

### 4.1. Study Design

This observational, retrospective, no-profit, monocentric cohort study examined data of 392 consecutive MBC patients treated between 2007 and 2017 at the Department of Oncology of the University Hospital of Udine (Italy). The study was conducted under the Declaration of Helsinki, and the Regional Ethics Committee approved the protocol (N° Protocol 14571 ratified in May 2018). Informed consent was obtained for the use of clinical data, rendered anonymous, for purposes of clinical research, epidemiology, training, and study of diseases.

### 4.2. Data Source

Clinicopathological information and blood sample data were collected from electronic health records. We defined MBC subgroups as follows: Luminal A (ER or PR positive, HER2-negative, Ki-67  $\leq 14\%$ ), luminal B (ER or PR positive, HER2-negative, Ki-67  $> 14\%$ ), luminal HER2 (HER2-positive and ER or PR positive), HER2-positive (ER and PR negative, HER2-positive), and triple negative (ER and PR negative, HER2-negative) [4].

### 4.3. Blood Sample Analysis

Serum LDH and ALP data were retrospectively evaluated. Blood samples data were eligible for review if performed within one month before first-line treatment administration (baseline pre-treatment sample) and after 12 weeks  $\pm 1$  week after first treatment dose (post-treatment sample). The quantitative determination of LDH and ALP was performed using the Roche Cobas 8000 c702 system (Roche Diagnostics, Indianapolis, IN, USA). The LDH and ALP cut-off value for normality was the normal upper limit (NUL) defined by the analytical system used (480 IU/L and 104 IU/L, respectively).

### 4.4. Statistical Analysis

The study was designed in order to explore the prognostic role of LDH response after 12 weeks of first-line treatment in unselected MBC, with a hierarchical design: The independent prognostic impact of plasmatic LDH was first evaluated at baseline and then for its variation at 12 weeks, using a multivariate Cox regression model for both PFS and OS with 95% confidence interval (95% CI). A two-sided  $p < 0.05$  was considered statistically significant. The multivariate model included the following covariates: The molecular profile, ECOG PS, number of metastatic sites, CNS, liver and bone localizations, and plasmatic ALP levels (at baseline and its variation at 12 weeks). Baseline clinicopathological characteristics were summarized through descriptive analysis. OS was defined as the time elapsed between the start of first-line treatment and death or last follow-up. PFS was defined as the interval between the start of first-line treatment and disease progression or death for any cause. Differences in survival were tested by a log-rank test and represented by Kaplan–Meier survival curves. Statistical analysis was performed with STATA (StataCorp, [www.stata.com](http://www.stata.com)) (2015) Stata Statistical Software: Release 14.2. College Station, TX: StataCorp LP).

## 5. Conclusions

LDH is a routinely used biomarker with a well-established prognostic role in several solid tumors and hematological malignancies. Our study confirmed that LDH is an independent prognostic factor also in MBC and explored its value as a dynamic biomarker. To the best of our knowledge, this is the first study to demonstrate that LDH response to first-line treatment significantly impacts both PFS and OS in unselected MBC patients. If validated in prospective studies, LDH could represent a cost-effective biomarker to stratify patient's prognosis, monitor treatment efficacy, and to implement treatment strategies in MBC.

**Author Contributions:** G.P. contributed to the concept and design of the study, acquisition of data, statistical analysis, and interpretation of data. Furthermore, he contributed to the drafting and revision of the article and the final approval of the version to be published. L.G. contributed to the design of the study, to statistical analysis, and to interpretation of data. S.Z. and F.C. contributed to LDH and ALP analytical evaluation and revised the article. C.L., Michele Bartoletti, L.B., and S.B. contributed to the drafting and revision of the article. D.B., M.G.V., V.F., and M.C. contributed to acquisition of data and revised the article. M.B., M.M., and G.F. contributed to the revision of the article. A.M.M. and F.P. contributed to the concept and design of the study, interpretation of data, revision of the article, and the final approval of the version to be published.

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Review

# Exercise Intervention Improves Clinical Outcomes, but the “Time of Session” is Crucial for Better Quality of Life in Breast Cancer Survivors: A Systematic Review and Meta-Analysis

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**Abstract:** This study examined the effects of exercise intervention on the quality of life (QoL), social functioning (SF), and physical functioning (PF) of breast cancer survivors, and identified the responsible and optimal exercise characteristics for amelioration of outcomes. Randomized controlled trials (RCTs) that adopted exercise intervention and measured the QoL, SF, and PF of breast cancer patients were included. We used meta-analysis to calculate the pooled effect, and meta-regression to identify the responsible exercise characteristics (type, frequency, duration, and time). Subgroup analysis assessed the optimal “time of session” for an improved QoL. The Cochrane risk-of-bias tool was used to determine the quality of studies. In the systematic review, we included 26 RCTs with a total of 1892 breast cancer patients, whilst 18 trials were considered for meta-analysis (exercise = 602; control = 603). The pooled effect showed that exercise intervention substantially improved the QoL (standardized mean difference (SMD) = 0.35;  $I^2 = 61\%$ ; 95% confidence interval (CI): 0.15–0.54;  $p = 0.0004$ ), SF (SMD = 0.20;  $I^2 = 16\%$ ; 95% CI: 0.08–0.32;  $p = 0.001$ ), and PF (SMD = 0.32;  $I^2 = 32\%$ ; 95% CI: 0.20–0.44;  $p < 0.00001$ ). Meta-regression analysis showed that improved QoL was associated ( $p = 0.041$ ) with the “time of session”. More specifically, sessions conducted for medium-time (>45 to ≤60 min;  $p = 0.03$ ) and longer-time (>60 to 90 min;  $p = 0.005$ ) considerably improved the QoL, whilst shorter-time (≤45 min;  $p = 0.15$ ) did not. To summarize, exercise interventions improved the QoL, SF, and PF of breast cancer survivors, where the “time of session” appeared to be crucial for an effective improvement in the QoL.

**Keywords:** physical activity; breast cancer survivors; physical function; social well-being; exercise characteristics

## 1. Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide. The number of newly diagnosed breast cancer patients reached 2.1 million in 2018, accounting for one quarter of female cancer cases [1]. Globally, the number of newly diagnosed breast cancer patients is predicted to reach 3.2 million by 2050 [2,3]. Currently, the highest incidence of breast cancer is found in the developed regions, including Western Europe, Northern Europe, Australia and New Zealand, and North America [1]. Owing to advancements in medical care and treatment, the number of post-treatment cancer survivors has also increased worldwide. The 5-year survival rate for breast

cancer patients is 90.2% in the USA, 89.5% in Australia, and 83.2% in China, while it is only 66.2% and 65% for patients in India and Malaysia, respectively [4]. Despite this, after treatment or prognosis, most cancer survivors suffer from a series of psychological and physical adverse symptoms, including nausea, insomnia, depression, anxiety, and fatigue. These side effects eventually impair the social functions (SF) and physical functions (PF) of the women, and lead to a decrease in their overall quality of life (QoL). For instance, negative outcomes, such as decreased expectations for the future, breakdown in social relations, limitation of daily activities, and a decline in self-care ability, were commonly observed in breast cancer survivors [5,6].

In recent decades, there has been a significant increase in the prescription of exercise to cancer survivors as a means to overcome treatment-induced adverse effects [7,8]. Based on the evidence from several randomized controlled trials (RCTs), the American College of Sports Medicine (ACSM) roundtable concluded that exercise training is safe both during and after treatment, and it can improve the QoL and PF, as well as reduce depression, anxiety, and fatigue in breast cancer survivors [9]. However, some studies using exercise intervention reported equivocal results in various clinical outcomes in breast cancer patients. For instance, an RCT of 500 breast cancer survivors reported no change in the QoL (all domains), depression, or fatigue after a 12 month exercise (60 min) program [10]. In contrast, another RCT showed that a 12 week supervised exercise program (>60 min) improved the functional and global health scores linked to the QoL, whilst home-based exercise (30 min) improved only the global health score of the QoL in breast cancer patients [11]. Moreover, resistance exercise (45 min, under an 8-week program) or aerobic exercise (60 min, under a 17-week program) interventions were found to be ineffective in improving the QoL in breast cancer patients [12,13]. To address the type and dose response of exercise (~16 weeks) on PF, Courneya et al. reported that neither high-intensity aerobic exercise (50 to 60 min) nor combined aerobic–resistance exercise (50 to 60 min) were superior to standard aerobic exercise (25 to 30 min) in breast cancer survivors [14].

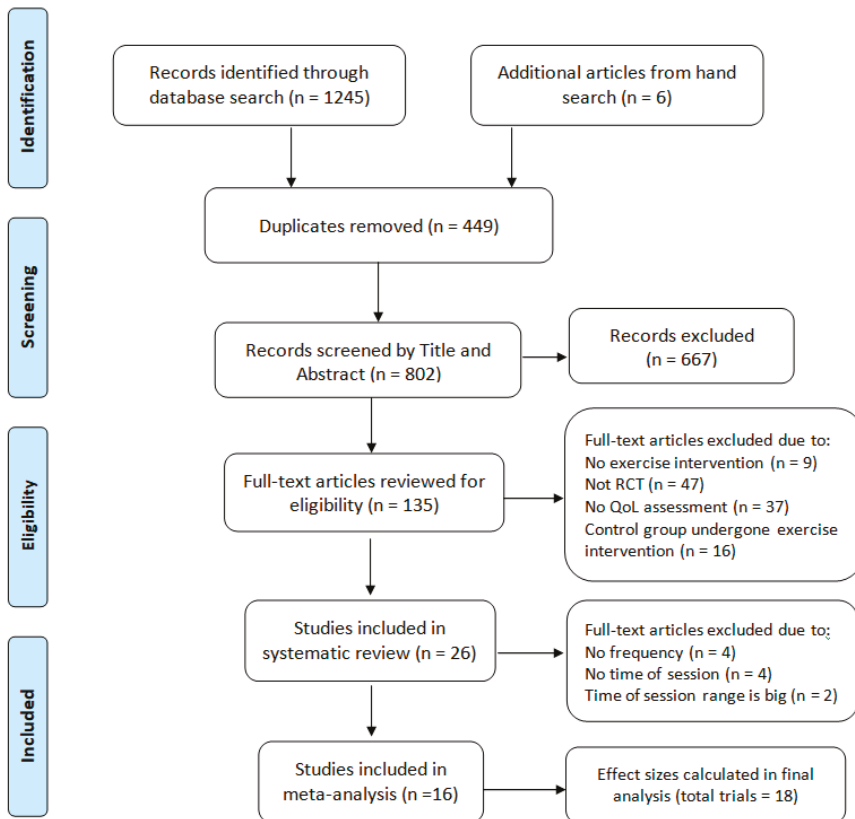
From these RCTs, it appears that the beneficial effects of exercise on the health outcomes (QoL, SF, PF) should be moderated using one or more exercise characteristics, such as frequency (F), intensity (I), type (T), and time (T) (i.e., the FITT factors). Therefore, to enhance the beneficial effects of exercise, it is necessary to better understand the influences of exercise variables on the QoL, SF, and PF of breast cancer patients. In this context, a systematic review and meta-analysis of 16 RCTs found that shorter workouts improved both the physical and social functioning, whilst longer workouts only improved the PF in patients with cancer. In addition, the optimal FITT factors to improve patients' QoL remained unanswered in that study [15]. A recent meta-analysis of 34 RCTs reported a significantly improved QoL and PF of breast cancer patients after exercise. However, it was claimed that these improvements were not moderated by the exercise characteristics [16]. Another meta-analysis of eight RCTs showed a non-significant improvement in the QoL from exercise intensity or any type of exercise in breast cancer patients [17]. In contrast, a recent systematic review of 36 trials concluded that all types of exercise interventions improved the QoL in patients with breast cancer. Combined exercise was specifically cited as being more efficient than aerobic or resistance exercise alone, although no meta-analysis data were provided [7].

These systematic reviews and meta-analyses attempted to delineate the influence of exercise characteristics on patients' clinical outcomes. However, none of these studies revealed the efficacy of individual exercise variables (i.e., type, frequency, duration, and time of session) on improving the QoL, SF, or PF of breast cancer survivors. Most importantly, the conclusions are debatable with regards to the optimal exercise variables on clinical outcomes. As such, in order to prescribe exercise intervention as an alternative medicine, it is crucial to identify the effective and optimal exercise variables that may effectively improve a patient's clinical outcomes. Therefore, in the current systematic review and meta-analysis, we seek to examine the effects of exercise intervention on the QoL, SF, and PF of breast cancer survivors. We further sought to explore the most effective exercise characteristics (type, frequency, duration, time, and total exercise time) using a meta-regression analysis, whilst the optimal dose of exercise (time) for an improved QoL was determined using a subgroup analysis.

## 2. Results

### 2.1. Search Results and the Selection of Studies

In the initial search, a total of 1251 articles were identified, with 1245 from electronic databases (PubMed, Web of Science, ScienceDirect, EMBASE, SportDiscus, Google Scholar) and six from a manual search (i.e., the reference list of included studies and other reviews). After removing 449 duplicates, 802 records were retrieved for further assessment. The titles and abstracts of the retrieved studies were screened using EndNote and a one by one reading, which also led to an exclusion of 667 articles. Then, the full-text of the remaining 135 articles was evaluated for the inclusion criteria, and 109 articles were excluded based on the reasons detailed in Figure 1. Finally, 26 articles that met our study’s criteria were included in the systematic review. Of these, articles with no information on exercise frequency [18–21], time of session [22–25], and with wide ranges in the time of session [26,27] were excluded. Finally, 16 studies possessing sufficient information regarding exercise characteristics and clinical outcomes were included in the meta-analysis. The detailed steps for article selection and the corresponding number of included and excluded articles in our study are presented as a flowchart in Figure 1.



**Figure 1.** Flowchart of the study selection according to the Preferred Reporting Items for the Systematic Review and Meta-Analysis (PRISMA) method.

## 2.2. Summary of the Included Studies

According to the criteria, 26 articles (RCTs) were included for the systematic review, and 16 of them were used for the meta-analysis. The RCTs were intercontinental, that is, from Australia, Canada, China, England, France, Germany, Italy, Kosovo, Spain, and the United States. All the participants (1892 in total) in these RCTs were women with breast cancer. The number of patients in the trials (exercise and control) ranged from 16 to 220, and their cancer stages were from 0 to IV. For the systematic review, the included studies had conducted various types of exercise interventions, including aerobic exercise [24,25,27–33], resistance exercise [33–36], a combination of both [18,22,23,37–40], yoga [19–21,41,42], and Qigong [26,43]. The details of the patients and exercise characteristics are presented in Table 1.

For the meta-analysis, we included 16 studies that contained 18 trials, with a total of 1205 patients (exercise = 602; control = 603). Of these studies, seven trials performed aerobic exercises [28–33], four trials performed resistance exercises [33–36], four trials underwent a combination of aerobic and resistance exercises [37–40], two trials practiced yoga [41,42], and one trial performed Qigong [43], with durations of 6 to 26 weeks. The frequency of exercise varied from 2 to 7 times per week, and the length of each exercise session ranged from 25 to 90 min.

## 2.3. Patient-Reported Clinical Outcomes and Scales

The RCTs included in our meta-analysis used well-validated questionnaires (scales) to measure the patient-reported clinical outcomes, such as the QoL, SF, and PF. For the assessment of the QoL, the European Organization for Research and Treatment of Cancer-quality of life questionnaire-C30 (EORTC-QLQ-C30) was used in five studies [33–35,38,39], the functional assessment of cancer therapy-general (FACT-G) scale was adopted in eight studies [28,30,32,36,37,40,42,43], the functional assessment of cancer therapy-breast (FACT-B) scale was used in two studies [29,31], and the short form-36 (SF-36) scale was employed in one study [41]. Further information on the scales used to measure the SF and PF of breast cancer survivors is presented as supplementary data (Tables S1 and S2).

Table 1. Characteristics of the included studies.

Study	Country	Age (Years)		Sample (n)		Cancer Stage	Exercise Type	Time of Session (min)	Frequency (t/wk)	Duration (Weeks)	Outcome
		Exercise	Control	Exercise	Control						
Ying et al. 2019 [26]	China	1n: <40 36n: 40–60 9n: >60	2n: <40 28n: 40–60 10n: >60	46	40	I–III	Qigong	S:60, H:20	7	26	QoL, PWB, S/FWB
De Luca et al. 2016 [37]	Italy	50.2 ± 9.7	46.0 ± 2.8	10	10	I–III	AE + RE	90	2	24	QoL
Galiano-Castillo et al. 2016 [38]	Spain	47.4 ± 9.6	49.2 ± 7.9	39	37	I–III	AE + RE	90	3	8	QoL, PE, SF
Hagstrom et al. 2016 [36]	Australia	51.2 ± 8.5	52.7 ± 9.4	19	15	I–III	RE	60	3	16	QoL, PWB, S/FWB
Lahart et al. 2016 [32]	England	52.4 ± 10.3	54.7 ± 8.3	40	40	I–III	AE	30	3–7	26	QoL, PWB, S/FWB
Casla et al. 2015 [22]	Spain	45.91 ± 8.21	51.87 ± 8.21	44	45	I–III	AE + RE	(nr)	2	12	QoL
Schmidt et al. 2015a [34]	Germany	52.2 ± 9.9	53.3 ± 10.2	45	32	I–III	RE	60	2	12	QoL, PE, SF
Schmidt et al. 2015b [33]	Germany	AE:56 ± 10.15 RE:53 ± 12.55	54 ± 11.19	AE:20 RE:21	26	(nr)	AE RE	60	2	12	QoL, PE, SF
Swisher et al. 2015 [31]	United States	43–65	36–71	13	10	I–III	AE	30	5	12	QoL, PWB, S/FWB
Murtezani et al. 2014 [30]	Kosovo	53 ± 11	51 ± 11	30	32	I–III	AE	25–45	3	10	QoL, PWB, S/FWB
Steindorf et al. 2014 [35]	Germany	55.2 ± 9.5	56.4 ± 8.7	76	72	0–III	RE	60	2	12	QoL, PE, SF
Chen et al. 2013 [43]	China	45.3 ± 6.3	44.7 ± 9.7	49	47	0–III	Qigong	40	5	6	QoL
Kwiatkowski et al. 2013 [23]	France	51.8 ± 8.7	52.3 ± 10.1	113	107	(nr)	AE + RE	(nr)	7	2	QoL, PE, SF
Reis et al. 2013 [27]	United States	54 ± 11.1	59 ± 10.7	12	17	(nr)	AE	20–60	3	12	QoL, PWB, S/FWB



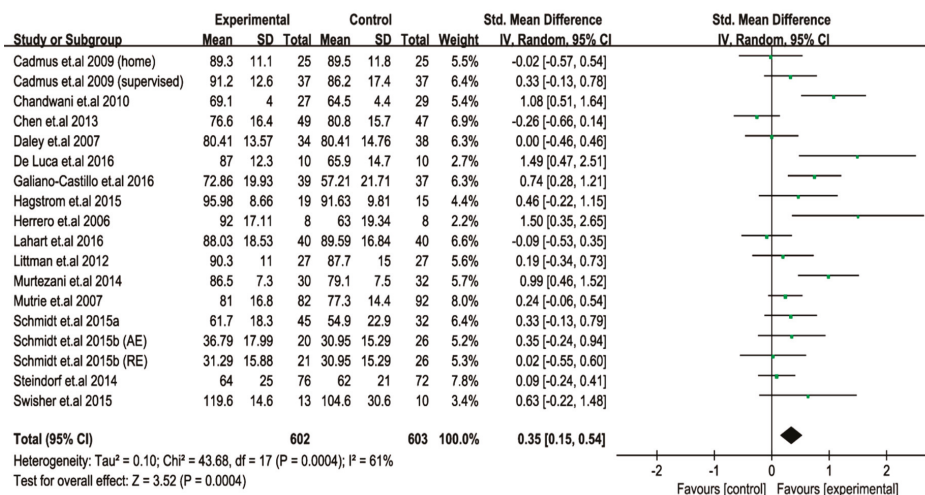
Table 1. *Contd.*

Study	Country	Age (Years)		Sample (n)		Cancer Stage	Exercise Type	Time of Session (min)	Frequency (t/wk)	Duration (Weeks)	Outcome
		Exercise	Control	Exercise	Control						
Litman et al. 2012 [42]	United States	60.6 ± 7.1	58.2 ± 8.8	27	27	0–III	Yoga	75	5	26	QoL, PWB, S/FWB
Chandwani et al. 2010 [41]	United States	51.39 ± 7.97	40.2 ± 9.96	27	29	0–III	Yoga	60	3	12	QoL, PF, SF
Haines et al. 2010 [18]	Australia	55.9 ± 10.5	59.5 ± 13.3	33	32	(nr)	AE + RE	(nr)	(nr)	24	QoL, PF, SF
Cadmus et al. 2009 [28]	United States	H:54.5 ± 8.2 S:56.5 ± 9.5	H:54 ± 10.9 S:55 ± 7.7	H:25 S:37	H:25 S:37	0–III	AE	30	5	26	QoL, PWB, S/FWB
Danhauer et al. 2009 [19]	United States	54.3 ± 9.6	57.2 ± 10.2	13	14	I–IV	Yoga	70	(nr)	10	QoL, PWB, S/FWB
Daley et al. 2007 [29]	England	51.6 ± 8.8	51.1 ± 8.6	34	38	(nr)	AE	50	3	8	QoL, PWB, S/FWB
Moadel et al. 2007 [20]	United States	55.11 ± 10.07	54.23 ± 9.8	45	26	I–III	Yoga	(nr)	(nr)	12	QoL, PWB, S/FWB
Mutrie et al. 2007 [40]	England	51.3 ± 10.3	51.8 ± 8.7	82	92	(nr)	AE + RE	45	3	12	QoL, PWB, S/FWB
Culos-Reed et al. 2006 [21]	Canada	51.18 ± 10.33		18	18	(nr)	Yoga	75	(nr)	7	QoL
Herrero et al. 2006 [39]	Spain	50 ± 5	51 ± 10	8	8	I–II	AE + RE	90	3	8	QoL
Sandel et al. 2005 [24]	United States	59.7 ± 9.8	59.5 ± 13.3	19	19	(nr)	AE	(nr)	1–2	12	QoL
Courneya et al. 2003 [25]	Canada	59 ± 5	58 ± 6	24	28	I–III	AE	(nr)	3	12	QoL, PWB, S/FWB

Note: t/wk, times/week; H, home-based; S, supervised; AE, aerobic exercise; RE, resistance exercise; AE + RE, combination of aerobic and resistance exercise; nr, not reported; QoL, quality of life; SF, social function; PF, physical function; PWB, physical well-being; S/FWB, social/family well-being.

### 2.4. Exercise Intervention Improves the QoL in Breast Cancer Survivors

The effect of exercise intervention on the QoL of breast cancer survivors was evaluated for 18 trials. The meta-analysis results showed that the change in QoL was extremely ( $p = 0.0004$ ) influenced by exercise intervention, with heterogeneity:  $\text{Tau}^2 = 0.10$ ;  $\text{Chi}^2 = 43.68$ ; degrees of freedom ( $\text{df} = 17$ ); and  $I^2 = 61\%$  (Figure 2). Then, a meta-regression analysis was performed to identify the effective exercise variables that improved the QoL. Information regarding exercise type, time of session, frequency, duration, and total exercise time was entered into the meta-regression analysis. In the results, we found that except for the “time of session”, all other exercise characteristics (type, duration, frequency, and total time) were not associated with a change in QoL of breast cancer survivors. However, the “time of session” was significantly ( $p = 0.041$ ) correlated with an improved QoL in breast cancer survivors following exercise intervention (Table 2).



**Figure 2.** Changes in the quality of life (pooled outcomes) after exercise intervention in patients with breast cancer. SD, standard deviation; IV, inverse variation; CI, confidence interval; df, degrees of freedom.

**Table 2.** Meta-regression analysis to identify the effective exercise moderators on an improved quality of life in breast cancer survivors.

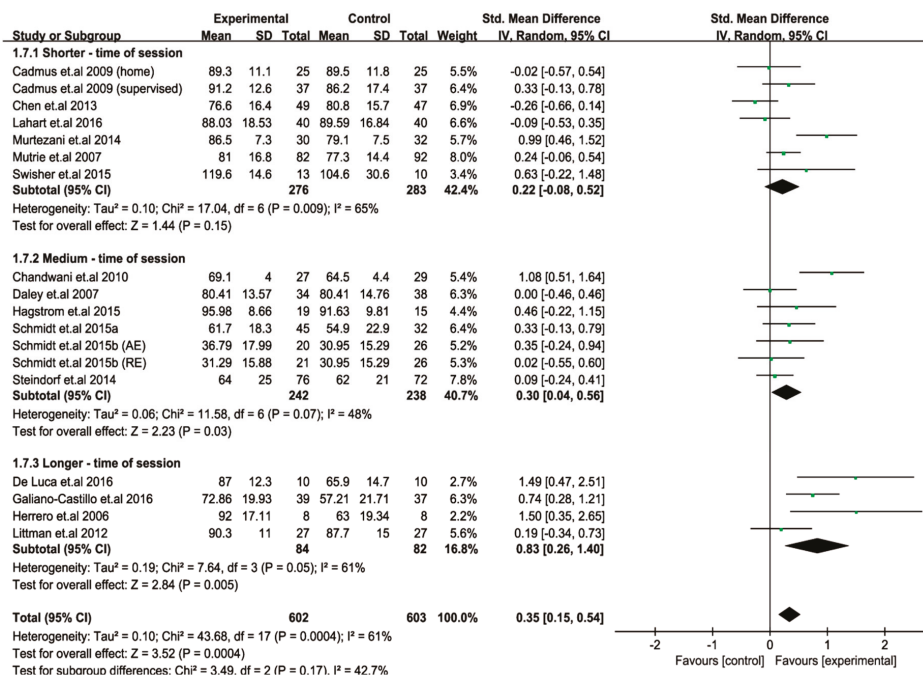
Exercise Characteristics	Coefficient	Standard Error	T Value	p-Value
Type of exercise	-0.3471926	0.3498219	-0.99	0.339
Time of session	0.0121459	0.0054789	2.22	0.041 *
Frequency	-0.1384031	0.0839449	-1.65	0.119
Duration	-0.006877	0.0171193	-0.40	0.693
Total time of exercise	-0.0000111	0.0000583	-0.19	0.851

\* Represents a significant correlation between the quality of life and exercise variable (time of session).

### 2.5. Longer Time of Session Profoundly Improves the QoL in Women with Breast Cancer

To identify the optimal “time of session” for an improved QoL, the trials with “time of session” data were categorized into three subgroups, that is, shorter-time of session ( $\leq 45$  min; 7 trials), medium-time of session ( $> 45$  to  $\leq 60$  min; 7 trials), and longer-time of session ( $> 60$  to 90 min; 4 trials). In the subgroup analysis, we found that both the sessions over the medium-time (standardized mean difference (SMD) = 0.30;  $I^2 = 48\%$ ; 95% confidence interval (CI): 0.04 to 0.56,  $p = 0.03$ ) and longer-time (SMD = 0.83;  $I^2 = 61\%$ ; 95% CI: 0.26 to 1.40,  $p = 0.005$ ) significantly improved the QoL of the breast

cancer patients. However, a shorter-time of session showed no significant improvement in the QoL (SMD = 0.22;  $I^2 = 65\%$ ; 95% CI:  $-0.08$  to  $0.52$ ,  $p = 0.15$ ). Furthermore, patients that engaged in longer exercise sessions ( $>60$  to  $90$  min) appeared to achieve greater improvements (a bigger effect size) to their QoL, compared to the medium-time of session ( $>45$  to  $\leq 60$  min) (Figure 3).



**Figure 3.** Forest plot of the quality of life (QoL) changes (subgroup analysis) with different times of exercise sessions in patients with breast cancer. SD, standard deviation; IV, inverse variation; CI, confidence interval; df, degrees of freedom.

### 2.6. Beneficial Effects of Exercise on SF amongst Breast Cancer Survivors

Cancer or cancer treatment affects the social functioning of women with breast cancer. To address the influence of exercise on the SF, we included 15 eligible trials consisting of 1073 breast cancer patients (535 exercise; 538 control), and a meta-analysis was also performed. The test for the overall effect revealed that exercise interventions substantially ( $p = 0.001$ ) improved SF in women with breast cancer. The SF outcome extremely favored exercise, citing the SMD = 0.20,  $I^2 = 16\%$ , and 95% CI: 0.08 to 0.32. Our data further indicated that none of the other specified exercise variables were correlated with improving SF in female patients (Figure 4).

### 2.7. Beneficial Effects of Exercise on PF amongst Breast Cancer Survivors

A total of fifteen trials addressed the effects of exercise on the PF of female breast cancer survivors. The pooled outcome of meta-analysis also showed that exercise interventions improved the PF of breast cancer survivors ( $p < 0.00001$ ). The pooled SMD of the enhanced PF was 0.32 (0.20 to 0.44), at a 95% CI, and where the  $I^2$  was 32% after the interventions (Figure 5).

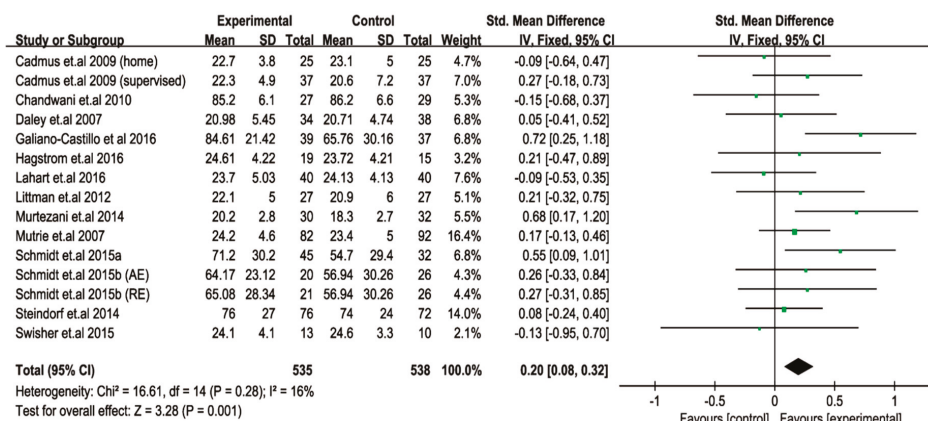


Figure 4. Pooled outcome of the changes in social function after exercise intervention in patients with breast cancer. SD, standard deviation; IV, inverse variation; CI, confidence interval; df, degrees of freedom.

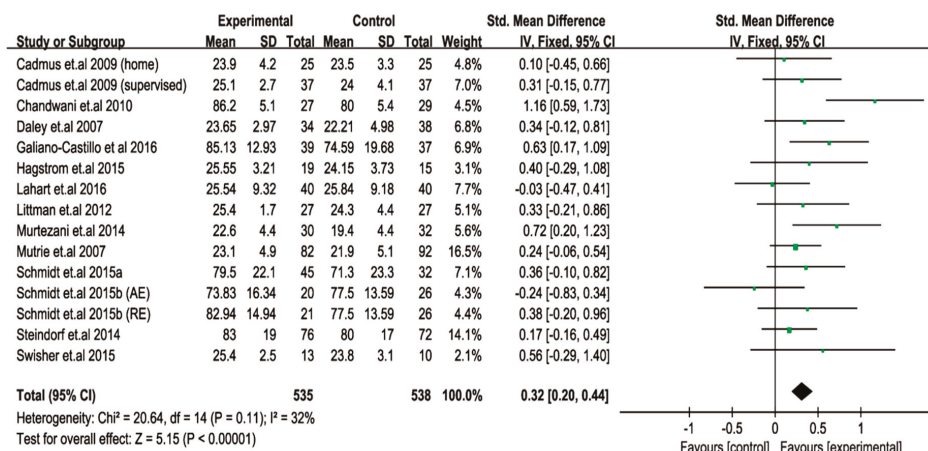


Figure 5. Pooled outcome of changes in physical function after exercise intervention in patients with breast cancer. SD, standard deviation; IV, inverse variation; CI, confidence interval; df, degrees of freedom.

### 2.8. Risk of Bias of Included Studies

The risk of biased judgments in the 26 articles is shown in Figure 6. For the selection bias, most of the studies (i.e., 20 trials) reported a low risk of random sequence generation, and seven studies were judged to have a high risk of allocation concealment. Several studies (i.e., 18 trials) were judged to have a high risk of bias in the blinding of participants towards exercise performance. Specifically, in the studies with exercise interventions, it may not be feasible to blind patients in the participation of exercise. It is stated that reporting such a high risk of performance bias does not necessarily compromise the study quality [44]. Nevertheless, we identified seven trials with detection bias and six trials with an attrition bias. Only four studies were judged to have a selective reporting bias. These results indicated that most of the included studies were not found to possess a high risk of selection, adherence, attrition, and reporting bias.

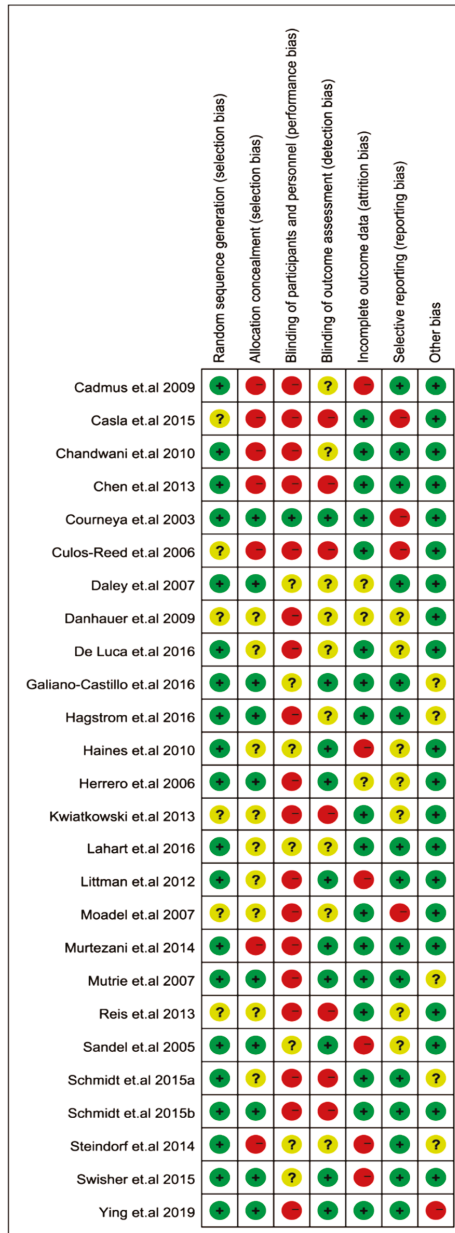


Figure 6. Risk of bias summary of the included studies. Green indicates a low risk of bias (+), red indicates a high risk of bias (-), and yellow indicates unclear risk (?).

### 3. Discussion

For the first time, our systematic review and meta-analysis demonstrated the influence of exercise intervention on the QoL, SF, and PF in female breast cancer survivors, and the “time of session” was identified as a crucial exercise variable for an improved QoL. Previous meta-analyses have stated that

exercise promotes a better QoL in breast cancer survivors; however, the intervention effect concerning the exercise type, frequency, duration, or time of session was inconclusive [15–17]. In this study, we included 26 RCTs and addressed the efficacy of exercise characteristics on the patient-reported clinical outcomes. Pooled outcomes revealed that the exercise intervention significantly improved the QoL, SF, and PF of breast cancer survivors. Our subsequent meta-regression analysis showed that an improved QoL was significantly correlated with the “time of session” and not with other exercise variables (i.e., type, frequency, duration, and total exercise time). Specifically, exercise sessions for a longer-time (>60 to 90 min) appeared to be superior to the sessions for a medium-time (>45 to ≤60 min) in improving the QoL, whilst shorter-time sessions (≤45 min) had no significant effect on the QoL.

Despite the incidence of breast cancer, the number of survivors has increased [4] owing to advanced medical care and facilities. However, this greater number of survivors are still suffering from treatment-induced adverse effects, such as impairments to social functioning, physical functioning, and QoL [5,6]. In post-treatment survivors, poor subjective well-being and impaired health-related QoL, along with extreme financial distress, could be vital risk factors of mortality [45]. A study on the Brazilian female population revealed that physical inactivity was responsible for more deaths (~12%) due to breast cancer, whilst other modifiable risk factors contributed for ~5% of deaths [46]. Evidence from a cohort study emphasized the importance of exercise in the reduction of all-cancer mortality, and it was reported to extend lifespan by ~3 years in both women and men [47]. In general, exercise interventions are aimed at improving the health-related physical fitness and overall well-being of cancer survivors.

Exercise prescription has gained significant attention in recent decades as a way to overcome treatment-induced adverse effects and to promote overall well-being. Several trials have adopted various exercise protocols, and these trials have demonstrated the practical applications of such interventions in improving the clinical outcomes in breast cancer survivors [31,37,48]. Prior to establishing this evidence, patients receiving cancer treatment were advised to stay rested and to avoid physically challenging activities that may cause additional burden and fatigue, instead of alleviating the cancer-related fatigue [49]. This conceptual notion was eventually ruled-out as studies revealed the beneficial effects of exercise interventions on the improved physiological and psychological domains of cancer survivors. In our systematic review, we synthesized the data from 26 RCTs that adopted various types of exercise patterns and with different frequencies, durations, or time of session. We found that exercise intervention significantly improved the clinical outcomes, including the QoL, PF, and SF in breast cancer survivors. Although the overall response of the outcomes towards the exercise interventions was favorable, the adopted exercise protocols in the RCTs were dissimilar in their type [26,33,38,42], duration [32,43], time and dose [30,39] or frequency [28,33]. Therefore, identification of the responsible exercise moderators and the optimal time and dose of exercise is required in order to shed light on the best exercise prescription for breast cancer survivors.

QoL is a subjective and multidimensional outcome, encompassing physical function, psychological state and emotional well-being, and social well-being [50]. Much has been done to address the influence of exercise interventions on a patients’ QoL, and several studies have also found either an unchanged or slightly improved QoL in breast cancer survivors. This inconsistent result in the QoL outcome from exercise interventions might be linked to several factors associated with the exercise characteristics, such as the type, frequency, duration, or time of session [10,26,28,51]. In our meta-analysis, the pooled outcome of 18 trials showed a profoundly improved QoL following exercise, where the heterogeneity was high. Similarly, a previous meta-analysis of 34 RCTs reported an improved QoL with exercise, although this improvement was not moderated by the exercise type or any other exercise characteristics [16]. Another meta-analysis of eight RCTs found a non-significant improvement in the QoL, linked to the intensity or type of exercise [17]. On the other hand, the practice of conventional exercises, such as yoga and qigong, was also reported to improve the QoL and well-being of female breast cancer survivors [26,41,48]. It indicates that either the type or duration of exercise could

influence the QoL scores in breast cancer survivors, although the optimal duration for this promising amelioration is still inconclusive.

The strength of our study was that we identified the “time of session” as a promising variable, which we considered to be responsible for the improved QoL in female breast cancer survivors. Our meta-regression analysis revealed a significant correlation between the “time of session” and an improved QoL, whereas other variables (i.e., type, frequency, duration, and total exercise time) were not associated with the QoL. A further subgroup analysis demonstrated that a shorter-time of session ( $\leq 45$  min) did not contribute to better a QoL. However, both the medium-time ( $>45$  to  $\leq 60$  min) and longer-time of sessions ( $>60$  to 90 min) profoundly improved the QoL, where longer-time sessions showed a bigger effect size. In contrast to our findings, a prior meta-analysis found that exercise intervention durations of  $\leq 45$  min (five trials) and  $>45$  min (three trials), had improved the QoL in patients with mixed types of cancer [15]. Another study showed that a longer time (higher dose) of either aerobic or combined exercise (50 to 60 min) was not superior to a standard aerobic exercise (25 to 30 min) program, in terms of the physical functioning of breast cancer survivors [14]. Another meta-analysis reported that neither the exercise time nor its duration significantly influenced the QoL of patients with cancer [16]. Similarly, a recent meta-analysis showed an improved QoL in cancer patients after supervised exercise, although no statistical difference was noted for the other exercise variables, including time of session [8]. Nevertheless, our findings were in agreement with the recommendations of the European Society for Clinical Nutrition and Metabolism (ESPEN), which cite that cancer patients should be engaged in exercise sessions for 45 to 60-min per day [52]. Therefore, to achieve the maximum benefits from exercise interventions, breast cancer patients should participate in any type of exercises for  $>45$  min per session.

To the best of our knowledge, this is the first systematic review and meta-analysis to address the effects of exercise on the SF of female breast cancer survivors. Despite the etiology of cancer and cancer treatment, there is still a limited understanding of cancer-related consequences, especially of patients’ social domains. Either the diagnosis or treatment can cause sequential damages to the social functioning of women with breast cancer [53]. In the overall health-related QoL, family well-being and social well-being are the most considerable social domains for female breast cancer patients. Therefore, it is necessary to study the impacts of cancer on the family and social relationships following diagnosis, treatment, and during survival [54]. Group exercise interventions were said to foster an improved overall QoL in survivors, because the group exercises provided access to other survivors, and therefore could address the issues related to stained social relationships, stigma, and isolation [55]. In view of this, a meta-analysis using mixed types of cancer reported improved SF in cancer patients after exercise interventions. To be specific, the improved SF was observed with more exercise frequency (3–5 times/week) and with shorter workouts, but this effect was not observed with longer workouts [15]. Another meta-analysis of nine trials attempted to address the association between exercise and social well-being in breast cancer survivors. However, we noticed a discrepancy between that study’s explanations and the pooled data. In addition, the role of exercise variables was not fully disclosed and the data were not extracted from several eligible studies [51]. In our meta-analysis, we included all eligible studies (15 trials) and we found an extremely improved SF after exercise. However, this beneficial effect might not be correlated with the exercise characteristics, based on the heterogeneity.

Next, we found that exercise interventions substantially improved the PF of female cancer survivors. Previous meta-analyses have also indicated that physical activity was correlated with an improved PF and decreased fatigue symptoms in breast cancer survivors [17,56]. A recent meta-analysis by Juvet and colleagues [57] concluded that there were improvements to the PF in breast cancer trials with exercise intervention. The study’s subgroup analysis for aerobic, resistance, and mixed interventions showed no significant group differences. However, the greater beneficial effects of mixed interventions appeared to be inconsistent with the data provided in their study [57]. In another meta-analysis, the improved physical fitness of cancer patients was said to be connected

with their exercise frequency (i.e., 5 times/week) and the duration of each intervention (shorter and longer) [15]. However, with a relatively higher number of studies (15 trials), we found no involvement of exercise characteristics on the improved PF. Furthermore, the pooled outcome from our study was consistent with previous meta-analyses and RCTs, which affirmed the benefits of exercise on an improved PF in cancer survivors. It is interesting to note that improved PF in breast cancer survivors was not superior with a higher dose of aerobic or combined exercise as compared to a standard dose of aerobic exercise [14]. Moreover, other meta-analyses have revealed that an improved PF was not moderated by the patient's demographic, clinical, and exercise characteristics or the FITT factors [8,16]. Considering these points, in order to maximize the benefits of exercise interventions on PF, the type of exercise with the most optimal frequency, duration, or time still needs to be further established.

#### *Significance of Exercise Intervention, Scales, and the Included Trials*

To address the importance of the “time of session” in cancer survivors, one study showed an improved QoL with both a shorter and longer “time of sessions” [15], whilst another study claimed no influence of “time of session” on the QoL [16]. Our study found that the “time of session” is primarily associated with an improved QoL in breast cancer survivors. The improved PF, SE, and overall QoL in cancer survivors following exercise intervention could be explained by several possible aspects. Exercise has been shown to increase the lean body mass of breast cancer patients, and decrease the fat percentage, body mass index, and insulin level with improved muscle strength and cardiopulmonary functions [25,51]. Moreover, by participating in group or supervised exercise programs, patients can interact with the researcher or other patients that are facing similar issues. This setting allows patients to obtain health advice on their overall well-being, and it also helps to decrease their sense of isolation, social stigma, as well as improve their self-esteem [55,58]. The clinical outcomes reported in our study were self-reported and measured using various scales, including the EORTC-QLQ-C30, FACT-G, FACT-B, and SF-36. These self-reported questionnaires and scales are designed to provide robust and meaningful measurements, which are determined by objectivity, reliability, and validity. All the scales used in the included trials were well-validated, feasible, and are widely accepted in cancer clinical trials [59–62]. The empirical evidence from previous studies and the data from our meta-analysis emphasized that the implementation of exercise interventions is crucial in ameliorating the clinical outcomes in breast cancer survivors. Based on the location of the included studies, it might not be irrelevant to state that most of these RCTs were from Europe and North America. Therefore, we assume that this observation not only indicates the higher breast cancer survival rates in those regions, but it also implies the need for significant efforts and studies targeted towards the Asian region, where post-treatment cancer rehabilitation is relatively lower than the incidence.

## **4. Materials and Methods**

### *4.1. Search Strategy and the Identification of Studies*

Comprehensive article searches were performed using the PubMed (Medline), Web of Science, ScienceDirect, EMBASE, SportDiscus, and Google Scholar databases to identify the relevant articles (until February 2019). The keywords used for the article search were, “breast cancer”, OR “breast tumor”, OR “breast neoplasms”, OR “breast carcinoma”, AND “exercise”, OR “aerobic exercise”, OR “aerobic training”, OR “resistance exercise”, OR “resistance training”, OR “strength training”, OR “physical activity”, OR “kinesitherapy”, OR “motor activity”, OR “sports”, OR “yoga”, OR “Tai chi”, OR “Qigong”, AND “quality of life”, OR “QoL”, OR “outcomes”. The keywords denoting breast cancer and exercise were separately used again to search for studies with “social function” and “physical function” outcome measures. In addition, a manual search was conducted using the reference list of retrieved articles, systematic reviews, and meta-analyses to identify the relevant studies.



#### 4.2. Selection Criteria

Two investigators (Feng Hong and Weibing Ye) independently screened the titles and abstracts of all the identified articles, and the duplicates were removed. The full-text articles were then evaluated for inclusion in the systematic review and the meta-analysis. The inclusion criteria were as follows: (1) All the studies were RCTs; (2) the participants were adults diagnosed with breast cancer; (3) the experimental group (breast cancer patients) had undergone any type of exercise intervention, whilst the control group (breast cancer patients) had not undergone any exercise intervention; (4) the trials measured the QoL, PF, SF, or all these factors of the cancer patients; (5) the data of the outcomes was provided before and after the interventions; and (6) the study was published in English. The exclusion criteria were: (1) Inadequate statistical information or poor quality information; (2) insufficient information about the exercise characteristics; (3) the trials included other types of cancer patients or the studies were without a control trial; and (4) the data were provided as mean change within the trial.

Two of the authors (Feng Hong and Weibing Ye) carefully reviewed the studies, and then selected the articles that met the inclusion criteria. Then, a further in-depth review and additional information on the clinical outcomes and exercises was provided by other authors (Chia-Hua Kuo, Yong Zhang, and Yongdong Qian). Potential discrepancies regarding the inclusion or exclusion of the articles were discussed and resolved by another author (Mallikarjuna Korivi). The article review and selection process was performed using the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines [63], and a detailed flowchart was provided (Figure 1).

#### 4.3. Quality Assessment

The quality assessment procedure for the included RCTs was performed using the Cochran Collaboration risk of bias tool as in a previous study [64]. Each included study was evaluated for the source of bias, including: (1) random sequence generation and allocation concealment (selection bias); (2) blinding of study participants and personnel (performance bias); (3) blinding of outcome assessments (detection bias); (4) incomplete outcome data (attrition bias); (5) selective reporting (reporting bias); and (6) other sources of bias. The quality of each domain was rated as “low risk”, “high risk”, or “unclear”, and they were indicated using green (+), red (−), and yellow (?), respectively. The quality assessment of the trials was independently performed by two of the three researchers, and then compared (Feng Hong, Weibing Ye, and Yongdong Qian). The disagreements were resolved by discussing with another review author (Mallikarjuna Korivi).

#### 4.4. Data Extraction

Data from all the included trials were extracted by two independent authors (Feng Hong and Weibing Ye), and they were verified by another review author (Mallikarjuna Korivi). All the data were presented as mean with standard deviations (SD). Any data provided as standard error in the trials were converted to SD. Details of the included articles, such as authors, year, and country of publication, were recorded. Demographics of the patients (i.e., mean age, sample size, and cancer stage), as well as the characteristics of the exercise (i.e., type, time of session, frequency, duration, and total exercise time) were extracted from the included trials. The type of questionnaire used to determine the outcome measures (QoL, PF, SF) was also obtained, and the details are presented in Table S1.

#### 4.5. Outcome Measures

All the clinical outcomes, including the QoL (general health, global, and overall QoL), PF, and SF, were self-reported. The general health, PF, and SF subscales from the generic short-form 36 (SF-36) [61] were used as the measures for self-reported QoL, self-reported PF, and self-reported SF, respectively. The global QoL, PF, and SF scales from the disease-specific European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 questionnaire [65] and the cancer rehabilitation evaluation system short form [66] were used as the measures for self-reported QoL, self-reported

PF, and self-reported SF. The total scores from the functional assessment of cancer therapy (FACT-G, FACT-B) [62,67] were used as a measure for the self-reported QoL, whilst the physical well-being scale was used as a measure of the self-reported PF, and the social and family well-being scale was used as a measure of SF.

#### 4.6. Subgroup Division and Analysis

The identified trials with sufficient “time of session” data were categorized into three subgroups, including the shorter-time of session ( $\leq 45$  min), medium-time of session ( $>45$  to  $\leq 60$  min), and longer-time of session ( $>60$  to 90 min). This division was based on the time of each exercise session that the cancer patients performed during the course of intervention. The European Society for Clinical Nutrition and Metabolism (ESPEN) has recommended that cancer patients should engage in 45 to 60 min exercise sessions per day [59]. Based on the ESPEN recommendations and several other studies, we intended to examine the effects of shorter- and longer-times of exercise sessions on the clinical outcomes. Other exercise characteristics (i.e., type, frequency, duration, and total exercise time) were not correlated with the change of outcomes in patients with breast cancer.

#### 4.7. Statistical Analysis

The Cochrane Collaboration’s Review Manager (RevMan 5.3., Copenhagen, Denmark) program was used to analyze the effects of exercise characteristics on the clinical outcome measures (QoL, SF, PF) in breast cancer survivors. Owing to the different measurement scales, we calculated the standardized mean difference (SMD) at 95% confidence intervals (CI). The  $I^2$  statistic was reported as an indicator of heterogeneity, with  $I^2 \geq 50\%$  representing high heterogeneity and  $I^2 < 50\%$  representing low heterogeneity. If the heterogeneity was low, the fixed-effects model was used for the meta-analysis. If heterogeneity was high, then the random-effects model was used for the meta-analysis. Based on the heterogeneity significance (pooled outcome), meta-regression analysis was performed to identify the correlations between the exercise characteristics (type, time, frequency, duration, and total exercise time) and the outcome measures (QoL, SF, PF) of breast cancer patients. We used the STATA version 12 (StataCorp, College Station, TX, USA) for the meta-regression analysis. In the analysis, the exercise “time of session” was found to be associated with an improved QoL. Hence, the eligible trials containing “time of session” data were assigned into three subgroups, as described above. Then, the subgroup analysis was conducted to identify the effective “time of exercise session” to improve the QoL in female breast cancer patients.

## 5. Conclusions

Our findings convincingly demonstrated that exercise intervention (of any type) is beneficial to improving the QoL, SF, and PF of female breast cancer survivors. However, the improved QoL from exercise intervention was specifically associated with the length of the exercise session. Participation in the exercise sessions for more than 45 min (medium- or longer-time) effectively improved the QoL, whilst a shorter-time of session ( $<45$  min) did not contribute to a significant improvement. Our findings suggest that the “time of session” could be the most decisive factor in improving the overall QoL in cancer survivors. Therefore, prescribing exercise programs with  $>45$  min per session would be a promising approach to promoting the overall health-related QoL of breast cancer survivors.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6694/11/5/706/s1>, Table S1: Characteristics of the 18 trials, Table S2: Assessment scales and studies.

**Author Contributions:** Conceptualization, W.Y., Y.Q., and M.K.; methodology, W.Y., M.K., and F.H.; validation, W.Y., Y.Q., and M.K.; formal analysis, F.H., and Y.Q.; investigation, F.H., W.Y., and M.K.; resources, C.-H.K. and Y.Z.; data curation, F.H., W.Y., and M.K.; writing—original draft preparation, F.H. and W.Y.; writing—review and editing, M.K.; visualization, C.-H.K., W.Y., and M.K.; supervision, W.Y. and M.K.; project administration, W.Y. and Y.Q.; funding acquisition, W.Y.

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