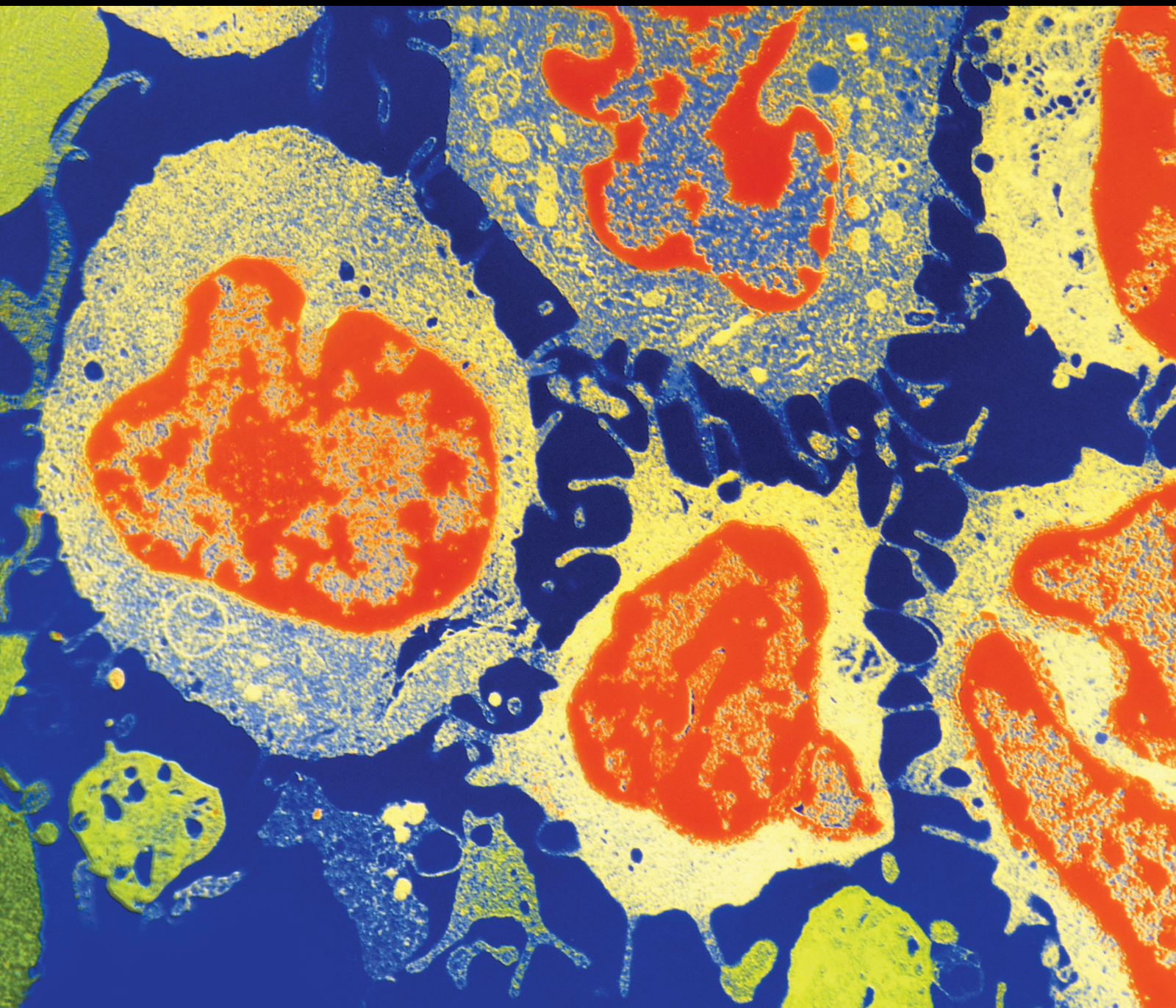# Biomarkers for Breast Cancer Research, Diagnosis, and Treatment

Lead Guest Editor: Arran K. Turnbull
Guest Editors: Cigdem Selli and Mike Dixon

Biomarkers for Breast Cancer Research, Diagnosis, and Treatment

Biomarkers for Breast Cancer Research, Diagnosis, and Treatment

Lead Guest Editor: Arran K. Turnbull
Guest Editors: Cigdem Selli and Mike Dixon

Contents

Clinical Study

Effectiveness of a Global Multidisciplinary Supportive and Educational Intervention in Thermal Resort on Anthropometric and Biological Parameters, and the Disease-Free Survival after Breast Cancer Treatment Completion (PACThe)

Marie-Paule Vasson [1,2] **Fabrice Kwiatkowski,**[3,4] **Adrien Rossary,**[2] **Sylvie Jouvency,**[1] **Marie-Ange Mouret-Reynier,**[5] **Martine Duclos,**[6] **Isabelle Van Praagh-Doreau,**[5] **Armelle Travade,**[7] **and Yves-Jean Bignon**[3]

[1]*Jean Perrin Comprehensive Cancer Centre, Department of Nutrition, 58 Rue Montalembert, 63011 Clermont-Ferrand, France*
[2]*University of Clermont Auvergne, INRA, UMR 1019 Human Nutrition Unit, CRNH-Auvergne, 28 Place Henri Dunant, 63000 Clermont-Ferrand, France*
[3]*Jean Perrin Comprehensive Cancer Centre, Department of Oncogenetics, 58 Rue Montalembert, 63011 Clermont-Ferrand, France*
[4]*University of Clermont-Auvergne, Laboratory of Mathematics, Probabilities and Applied Statistics, 28 Place Henri Dunant, 63000 Clermont-Ferrand, France*
[5]*Jean Perrin Comprehensive Cancer Centre, Department of Oncology, 58 Rue Montalembert, 63011 Clermont-Ferrand, France*
[6]*Gabriel Montpied University Hospital, Department of Sport Medicine and Functional Explorations, 58 Rue Montalembert, 63000 Clermont-Ferrand, France*
[7]*Centre République, Department of Senology, 99 Avenue de La Republique, 63100 Clermont Ferrand, France*

Correspondence should be addressed to Marie-Paule Vasson; m-paule.vasson@uca.fr

A growing knowledge highlights the strong benefit of regular physical activity in the management of breast cancer patients, but few studies have considered biological parameters in their outcomes. In the prospective randomised trial after breast cancer treatment completion "PACThe," we determined the effects of physical activity and nutritional intervention on the biological and anthropometric status of patients after one year of follow-up, and clarified the link between biomarkers at allocation and disease-free survival. 113 patients from the population of the "PACThe" study ($n = 251$) were analysed for biological parameters. Patients were randomized after chemotherapy in two arms: the intervention "SPA" receiving a 2-week session of physical training, dietary education, and physiotherapy ($n = 57$), and the control "CTR" ($n = 56$). Diet questionnaire, anthropometric measures, and blood parameters were determined at allocation and one year later. Survival and recurrence were checked over 7 years. Data were considered as a function of BMI, i.e., ≤25 for normal, 25–30 for overweight, and >30 for obese patients. At allocation, the large standard deviation for nutrient-intake values reflected an unbalanced diet for some patients in the three groups. At one-year follow-up, we noticed an increase in glucose ($p < 10^{-6}$), insulin ($p < 10^{-7}$), and adiponectin ($p < 0.022$) plasma levels for both intervention arms, which were more accentuated for the >30 groups. Using the Cox model, we demonstrated that the highest testosterone plasma values were linked to an increase of the recurrence risk (HR [CI–95%] = 5.06 [1.66–15.41]; $p = 0.004$). One-year after a global multidisciplinary supportive and educational intervention, we found few anthropometric and biological changes, mainly related to the patient's initial BMI. We highlighted the importance of plasma testosterone in the evaluation of patient's recurrence risk. Future studies would help better understand the mechanisms by which such multidisciplinary interventions could interact with breast cancer recurrence and define the most effective modalities.

1. Introduction

Over many years, growing knowledge has indicated the strong benefit of regular physical activity in the management of breast cancer patients [1]. Despite an extensive literature of clinical trials, data from these studies showed positive but modest effects, which may be underestimated due to great variability in the intervention strategies and intensity of monitoring [2, 3]. These interventions produce short-term changes in physical activity and patient behaviour, but data are scarce on recurrence and long-term follow-up. Some studies have highlighted long-term barriers to exercise after diagnosis of breast cancer, including psychological barriers (e.g., low motivation and dislike of gym), environmental barriers (e.g., employment priority and low access to facilities), and lack of time [4]. Regarding the large variability of practice procedures, further research is required to investigate how to sustain positive effects of exercise over time and to determine essential attributes of exercise (mode, intensity, frequency, duration, and timing) by cancer type and cancer treatment for optimal effects [5]. The introduction of wearable activity monitors into cancer care could improve the understanding of the association between physical activity and patient behaviour, as previously suggested [1].

Moreover, analyses are needed to provide insight into how physical activity interventions work. Such studies should accelerate the identification of effective behaviour changes and permit the development of evidence-based practice with better standardisation. Currently, the mechanisms by which physical activity mediates its benefits remain unclear [6]. Most hypotheses regarding the biological pathways have focused on the impact of obesity on breast cancer risk and recurrence. In that field, the main research axes are, first, the implication of sex hormones, including both oestrogens and androgens (testosterone) [7]; second, the implication of metabolic hormones, such as insulin/insulin-like growth factor (IGF) axis and adipocytokines (leptin and adiponectin) [8]; and third, the implication of inflammatory factors (C reactive protein, CRP) [9]. None of these axes has clearly demonstrated efficiency in clinical trials, despite evidence of increased quality of life (QoL), reduced body weight in obese patients, and reduced recurrence.

The majority of studies that investigate the benefits of physical activity and nutritional interventions in breast cancer focus on weight loss, cardiorespiratory capacity, QoL, and overall well-being [5, 10, 11], but few of them considered the biological parameters of the patients in their outcomes [12, 13].

Taking into account these data and the interactions between physical activity and BMI, we performed a prospective randomized trial "Programme of Accompanying women after breast Cancer treatment completion in Thermal resorts" (PACThe) for complete-responder breast cancer patients after chemotherapy. In this trial, we demonstrated that the 2-week intervention durably influences the QoL of breast cancer patients after both short-term [14] and long-term treatment [15]. In the present study, we determined the effects of PACThe intervention on the biological and anthropometric status of patients after one-year follow-up and the link between the biomarkers and disease-free survival with seven years of follow-up after completion of breast cancer treatment.

2. Patients and Methods

2.1. Participants. Two hundred and fifty-one nonmetastatic breast cancer patients were enrolled between 2008 and 2010, as previously described [14]. The main inclusion criteria were notably invasive nonmetastatic breast carcinoma; less than 9 months after chemotherapy/radiotherapy completion, complete remission, $18.5 < BMI < 40 \, kg/m^2$, and written informed consent. Half of the 251 patients ($n = 113$) were investigated for biological parameters in the present study.

2.2. Study Design. Patients were randomized into two groups: "SPA," for the group attending the 2-week session in thermal centres, and "CTR," for the control group. The 2-week session performed in thermal centres included consultations with physicians, nutritionists, and psycho-oncologists; physical activity supervised by a physiotherapist for 2 h daily with endurance activities, strength training, and flexibility/stretching; SPA care consisting of bath, shower, and massage for half an hour per day; aesthetic care; and dietary meals with adapted menus, dietary education, and caloric intake limited to 1700–2000 kcal/day.

Besides standard oncological follow-up of the patients in the two groups, personal consultations with a dietician were organized to perform anthropometric measurements, provide dietary advice, and give encouragement for daily physical activity. Evaluation of survival/recurrence was made by patients' oncologist, with a follow-up period of 7 years [14]. The overall protocol design is available in a supplementary file.

2.3. Data Collection. Before randomization and at one year, the following analyses were performed on half of the population (SPA: $n = 57$; CTR: $n = 56$):

(1) Diet questionnaire

Dietitians evaluated oral intake based on a 72-h self-reported diet questionnaire.

(2) Body composition

Body weight was measured at each personal consultation. Lean body mass (LBM), fat mass (FM), and total body water were evaluated by multifrequency bioelectrical impedance analysis (Bodystat Quadscan 4000) using 5, 50, 100, and 200 kHz. Tricipital skinfold thickness was measured using a skin-fold caliper (Harpenden caliper). To assess central fat distribution, the waist circumference (WC) was evaluated to the nearest 0.5 cm using a standard tape measure placed between the lowest rib and the iliac crest, with the patient in the standing position. The hip circumference (HC) was estimated using a standard

tape measure placed horizontally at the widest point on the hip.

(3) Blood sampling and biological assays

Blood samples were collected at allocation and at one year. Plasma levels of biomarkers were determined as follows: glucose and HDL-cholesterol (colorimetry methods), C-reactive protein, and transthyretin (immunonephelometry) were determined at the biomedical laboratory of the recruiting centre; insulin and testosterone (ELISA) were determined at the hospital biochemistry laboratory (Clermont-Ferrand); IGF-1, leptin, and adiponectin (luminex) were determined at the Genotool platform (Toulouse); and CA 15-3 was determined at the anti-cancer centre radiobiology laboratory (Clermont-Ferrand).

(4) Recurrence follow-up

Disease-free interval was computed as months elapsed from date of randomization to documented breast cancer recurrence during seven years after breast cancer treatment completion. All recurrence types were considered, either local or distant (nodes, metastatis, and/or contralateral breast cancer).

2.4. Statistical Considerations.

Protocol design consisted of a multicentre parallel randomized prospective trial. Data were analysed using the intention-to-treat principle. Descriptive statistics are presented with mean ± standard deviation (SD) for Gaussian quantitative variables. Outcomes are shown with 95% confidence intervals. Categorical variables are described using counts by class and frequencies (%).

Comparison of outcomes per allocation group and per BMI class was tested with Student's t-test, one-way analysis of variance (ANOVA), or the Kruskal-Wallis H-test depending on homoscedasticity or normality of distributions. Two-way ANOVA was used to compare longitudinal variations between allocation groups, but without an interaction test because of unequal class sizes. Categorical data were compared with chi^2 test. To test the association between two quantitative parameters, Pearson's correlation coefficient was used, or Spearman's rank correlation if distributions were not Gaussian. Survival curves were drawn using Kaplan-Meier's method, and comparison of curves was performed using the Log-rank test. A backward and stepwise Cox proportional hazard regression model was used to perform the multivariate analysis of survival. Cutoff values of biological parameters to draw survival curves were chosen among quartiles of distribution.

All tests were two-sided and the nominal level of significance was 5%. Randomisation and statistics were performed using SEM software [16].

3. Results

Biological parameters were evaluated at allocation for half of the 251 patients: $n = 57$ for the "SPA" experimental group and $n = 56$ for the "CTR" control group (Figure 1). These 113 patients are referred to hereafter as the biological study population. At one year post-inclusion, 13 patients withdrew for familial or professional reasons, and 53 and 47 patients remained, respectively, for the SPA and CTR groups. The main covariates were distributed similarly between the allocation groups (Table 1). Cancer treatments were similar and standard for invasive tumours. Most patients' tumours were HR positive and treated using hormonotherapy, and a few (Her2+ tumours) using targeted therapy.

3.1. Diet, Body, and Biological Parameters at Allocation.

Results of the biological study population were considered in function of BMI scale and divided into three subgroups, i.e., ≤25 kg/m^2 for normal BMI, [25–30 kg/m^2] for overweight, and >30 for obesity (Tables 2 & 3). Overall diet mean results (Table 2) were within adult nutritional recommendations (17.3% ± 4.1, 46.7% ± 10.4, and 35.5% ± 8.6, respectively, for protein, carbohydrate, and lipid intakes). A large dispersion of values was observed, resulting in no significant difference between BMI subgroups except for total energy intake (TEI) ($p = 0.038$) and lipid intake in gram/day ($p = 0.034$). The large standard deviation for each nutrient-intake value reflected an unbalance diet for some patients in the three BMI subgroups.

All body parameters (Table 2) differed significantly by BMI subgroup ($p < 10 - 7$). As expected, the lean mass/fat mass ratio decreased with the BMI due to the expansion of the body fat mass, i.e., 2.4 ± 0.6, 1.7 ± 0.3, and 1.3 ± 0.3, respectively, for normal, overweight, and obese subgroups ($p < 10 - 7$).

As previously noticed, we observed a large dispersion of all biological parameter values (Table 3) regardless of BMI subgroup. Increased plasma levels of CRP ($p < 10 - 5$), insulin ($p < 10 - 4$), and leptin ($p < 10 - 7$) showed dysmetabolic disorders associated with overweight/obesity. As expected, the ratio of leptin/adiponectin significantly increased with BMI (0.53 ± 0.51, 1.26 ± 1.28, and 3.23 ± 3.86, respectively, for normal, overweight, and obese groups, $p < 10 - 7$). Conversely, a significant decrease in HDL-C level with BMI ($p < 10 - 4$) was observed. Transthyretin, similar between groups, was in the physiological range, showing no malnutrition disorders in the studied population. Other parameters (glucose, IGF-1, testosterone, and CA 15-3) were in the normal range, with no difference between BMI groups except for CA 15-3 ($p = 0.014$).

3.2. Changes in Diet, Body, and Biological Parameters One Year Later.

One year after inclusion, Diet consumption, body, and biological parameters of patients were reevaluated one year after inclusion. All the raw data are presented by BMI subgroups in two supplementary data files: one for the SPA group (Supplementary Table 1) and one for the CTR group (Supplementary Table 2). Variations in each parameter between inclusion and one-year follow-up are shown in Tables 4 and 5 and analyzed according to the intervention group (SPA effect), one-year follow-up (time effect), and BMI subgroups (BMI effect).
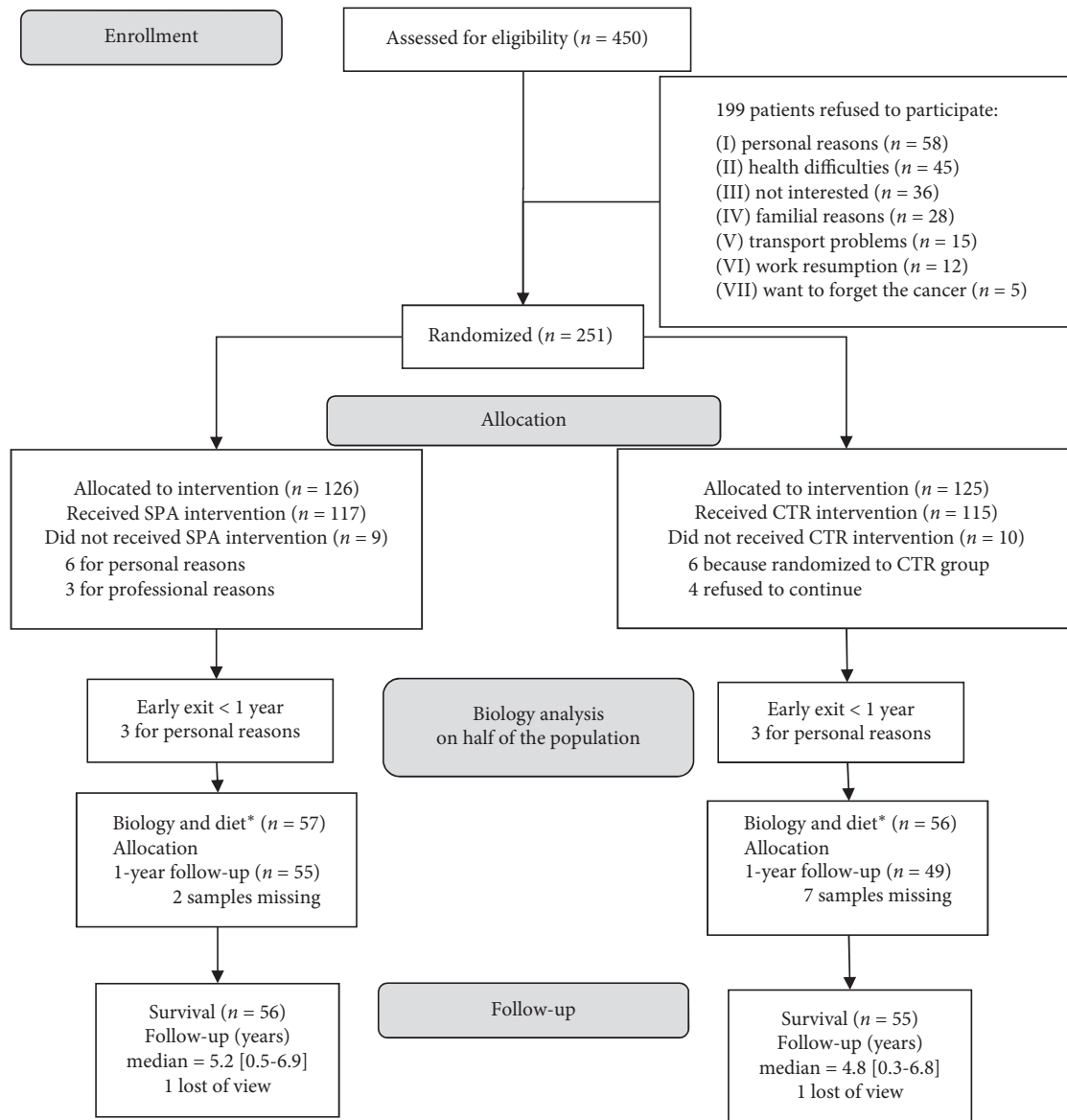
FIGURE 1: Allocation diagram and flow chart. *Diet, nutritional, and body data collection.

No significant difference was observed for diet parameters (Table 4) regardless of the intervention group, the time window, or the BMI subgroup, except for the total energy intake with time ($p = 0.039$). For the SPA group, total energy intake remained stable for BMI subgroups ≤25 and [25–30 kg/m^2], whereas a strong reduction (−400 kcal/d) in the BMI >30 subgroup led to both carbohydrate (−21.5%) and lipid (−13.8%) intake decreases without change in patients' weight. For the CTR group, total energy intake decreased for ≤25 and >30 BMI subgroups due to a reduction in protein, carbohydrate, and lipid intakes. However, an increase in the mean body weight of 1 kg was observed for each BMI subgroup (supplementary data), which was not significant because of the large dispersion of individual values.

For body parameters (Table 4), we observed that only the BMI effect was significant ($p < 10^{-7}$). All the parameters were significantly related to BMI but remained stable

considering both SPA and time effects. For the SPA and CTR >30 BMI subgroups, a reduction in brachial and abdominal circumferences tended to correlate with an increase in hip circumference.

No significant SPA effect was observed for biological parameters (Table 5), except for transthyretin ($p = 0.041$) and CA 15-3 ($p = 0.04$) plasma levels, although these remained in the normal ranges. For the time effect, a significant increase in both glucose ($p = 0.04$) and insulin ($p = 0.035$) and a decrease in HDL-C ($p = 0.027$) plasma levels were observed. As expected, several parameter variations were related to BMI in the two groups as previously shown at allocation. Notably, we noticed an increase in glucose ($p < 10 - 6$), insulin ($p < 10 - 7$), and adiponectin ($p = 0.022$) plasma levels regardless of the intervention group and more accentuated plasma levels for the >30 BMI subgroups. Conversely, a decrease in HDL-C plasma levels was observed ($p = 0.007$).

Table 1: Study population characterization.

Parameter	SPA group ($n = 57$) Size or mean ± SD (%) or [mini-max]	CTR group ($n = 56$) Size or mean ± SD (%) or [mini-max]	p value
Patients' age at allocation	52.0 ± 7.2 [36–66]	51.9 ± 10.6 [29–71]	0.97
Menopausal status	Yes = 33 (58%)	Yes = 35 (63%)	0.62
BMI—body mass index (kg/m^2)	25.4 ± 4.6 [18.4–35.9]	25.5 ± 4.4 [18.0–38.7]	0.92
BMI—class ≤25 kg/m^2	30 (53%)	27 (48%)	
25–30 kg/m^2	16 (28%)	22 (39%)	0.37
>30 kg/m^2	11 (19%)	7 (13%)	
SF36—global score/100	55 9 ± 15.2 [19.0–93.0]	56.8 ± 14.0 [29.0–95.0]	0.30
Surgery for breast cancer	Yes = 57 (100%)	Yes = 55 (98%)	0.50
Radiotherapy	Yes = 54 (95%)	Yes = 54 (96%)	0.98
Hormonotherapy	Yes = 43 (75%)	Yes = 43 (77%)	0.87
Herceptin	Yes = 5 (9%)	Yes = 7 (13%)	0.56
Chemotherapies: number of cycles	6.3 ± 1.1 [5–15]	6.0 ± 0.8 [3–9]	0.29

The main covariates of the studied population at allocation are presented with mean ± standard deviation (SD) for Gaussian quantitative variables. Outcomes are shown with 95% confidence intervals. Categorical variables were described using counts by class and frequencies (%). Comparison of outcomes was tested with Student's t-test or the Kruskal-Wallis H-test depending on homoscedasticity or normality of distributions. Categorical data were compared with the chi^2 test. All tests were two-sided, and the nominal level of significance was 5%.

Table 2: Diet and Body parameters at allocation.

Mean ± σ	All groups ($n = 113$)	BMI (kg/m^2)			p value of BMI effect
		≤25 ($n = 57$)	25–30 ($n = 38$)	>30 ($n = 18$)	
Diet parameters					
Total energy intake (TEI) (kcal/d)	1492 ± 450	1540 ± 358	1325 ± 378	1689 ± 678	0.038
Protein intake (g/d)	63.6 ± 20.2	65.3 ± 15.1	58.7 ± 20.0	68.8 ± 30.1	0.86
(% TEI)	17.3 ± 4.1	17.2 ± 3.5	17.9 ± 5.2	16.4 ± 3.1	0.71
Carbohydrate intake (g/d)	172.6 ± 61.5	175.3 ± 54.1	156.8 ± 53.7	197.2 ± 85.1	0.65
(% TEI)	46.7 ± 10.4	45.4 ± 9.5	48.1 ± 12.8	47.8 ± 6.5	0.75
Lipid intake (g/d)	59.7 ± 25.4	63.5 ± 22.3	50.6 ± 23.6	66.8 ± 31.8	0.034
% TEI	35.5 ± 8.6	36.8 ± 8.4	33.5 ± 9.8	35.8 ± 5.0	0.14
Body parameters					
Body weight (kg)	65.2 ± 12.5	56.6 ± 6.4	68.5 ± 5.8	85.3 ± 10.7	$<10^{-7}$
Lean mass (LM) (kg)	42.1 ± 5.8	39.6 ± 4.5	43.0 ± 4.8	47.9 ± 6.3	$<10^{-7}$
(%)	65.2 ± 6.8	69.6 ± 5.3	62.9 ± 3.7	56.3 ± 4.5	$<10^{-7}$
Fat mass (FM) (kg)	23.0 ± 7.8	17.2 ± 3.7	25.5 ± 3.1	36.2 ± 5.1	$<10^{-7}$
(%)	34.6 ± 6.7	30.1 ± 5.0	37.3 ± 3.8	43.1 ± 4.4	$<10^{-7}$
Ratio LM/FM	2.0 ± 0.6	2.4 ± 0.6	1.7 ± 0.3	1.3 ± 0.3	$<10^{-7}$
Cell mass (kg)	25.0 ± 4.0	22.8 ± 2.5	25.5 ± 3.3	30.7 ± 3.5	$<10^{-7}$
Total water (l)	32.9 ± 3.9	31.1 ± 2.6	33.2 ± 2.7	38.1 ± 4.6	$<10^{-7}$
(%)	51.3 ± 5.4	55.1 ± 4.0	48.5 ± 3.2	44.9 ± 2.8	$<10^{-7}$
Extracellular water (%)	24.3 ± 3.4	25.7 ± 1.7	23.1 ± 2.1	22.8 ± 6.5	$<10^{-7}$
Intracellular water (%)	27.1 ± 2.4	28.2 ± 1.8	26.1 ± 2.8	25.6 ± 1.2	$<10^{-7}$
Tricipital fold thickness (cm)	17.4 ± 8.6	12.5 ± 5.2	18.8 ± 7.2	29.6 ± 6.4	$<10^{-7}$
Arm circumference (cm)	30.2 ± 3.8	27.7 ± 2.2	31.1 ± 1.7	36.4 ± 3.3	$<10^{-7}$
Waist circumference (WC) (cm)	84.0 ± 13.5	75.4 ± 7.7	86.8 ± 9.0	105.5 ± 8.9	$<10^{-7}$
Hip circumference (HC) (cm)	101.1 ± 9.1	95.0 ± 4.9	103.5 ± 5.5	115.7 ± 5.6	$<10^{-7}$
Ratio WC/HC	0.83 ± 0.09	0.79 ± 0.07	0.84 ± 0.09	0.92 ± 0.08	0.000017

Diet parameters for food intake are expressed in raw value (gram/day) and in % of total energy intake. Body parameters are expressed in raw value (kilogram or liter) and in % of body mass. Comparison of outcomes per BMI group at allocation was tested with one-way analysis of variance (ANOVA). The test was two-sided, and the nominal level of significance was 5%.

We found significant positive correlations in the biological study population between leptin/adiponectin ratio and insulin ($r = 0.46$, $p < 10 - 7$) and CRP ($r = 0.46$, $p < 10 - 7$) and a negative correlation with HDL-C ($p = -0.46$, $p < 10 - 7$). The leptin/adiponectin ratio was strongly correlated with waist circumference ($r = 0.67$, $p < 10 - 7$), BMI ($r = 0.51$, $p < 10 - 7$), and cell mass ($r = 0.46$, $p < 10 - 7$). Moreover, despite the absence of variation in

TABLE 3: Biological parameters at allocation.

Mean ± σ	All groups (n = 113)	BMI (kg/m^2)			p value of BMI effect
		≤25 (n = 57)	25–30 (n = 38)	>30 (n = 18)	
Glucose (mmol/l)	5.2 ± 0.6	5.1 ± 0. 4	5.2 ± 0.6	5.6 ± 0.8	0.25
HDL-cholesterol (mmol/l)	2.13 ± 1.28	2.35 ± 1.35	1.98 ± 1.25	1.70 ± 0.97	0.0001
Transthyretin (g/l)	0.26 ± 0.04	0.26 ± 0.04	0.26 ± 0.04	0.26 ± 0.04	0.88
C-reactive protein (mg/l)	2.5 ± 3. 6	1.3 ± 1.2	3.2 ± 4.4	5.2 ± 4.9	0.000002
Insulin (mUI/l)	6.5 ± 6.2	4.7 ± 4.4	6.4 ± 4.4	12.1 ± 9.8	0.000013
IGF-1 (μg/l)	96.4 ± 49.3	95.8 ± 45.6	103.5 ± 45.7	84.7 ± 62.6	0.23
Leptin (μg/l)	5.7 ± 4.7	3.5 ± 2.6	6.0 ± 3.0	12.1 ± 6.0	<10^{-7}
Adiponectin (mg/l)	8.1 ± 5.1	8.9 ± 5.3	7.6 ± 4.8	6.6 ± 4.4	0.072
Leptin/adiponectin ratio	1.22 ± 2.02	0.53 ± 0.51	1.26 ± 1.28	3.23 ± 3.86	<10^{-7}
Testosterone (nmol/l)	0.82 ± 0.36	0.79 ± 0.29	0.83 ± 0.42	0.87 ± 0.38	0.67
CA 15-3 (kU/l)	18.1 ± 18.7	20.1 ± 24.5	14.1 ± 9.0	19.7 ± 8.4	0.014

Plasma biological parameters are expressed in usual unit per liter. Comparison of outcomes per BMI group at allocation was tested with one-way analysis of variance (ANOVA). The test was two-sided, and the nominal level of significance was 5%.

testosterone plasma level with SPA, time, or BMI effects, this parameter was significantly associated (i) positively with body weight ($r = +0.15$, $p = 0.03$), cell mass ($r = +0.19$, $p = 0.0072$), arm circumference ($r = +0.15$, $p = 0.026$), WC/HC ratio ($r = +0.15$, $p = 0.027$), and transthyretin ($r = +0.15$, $p = 0.028$) and (ii) negatively with TEI ($r = −0.16$, $p = 0.022$) and HDL-C ($r = −0.19$, $p = 0.007$).

3.3. Biological Parameters and Recurrence Relation.
We tested the association between biomarker plasma levels at allocation expressed in quartiles and the risk of recurrence during the seven-year follow-up. Highest HDL-cholesterol values were associated with the best survival without recurrence ($p = 0.047$). Conversely, the lowest testosterone and CA 15-3 values were associated with longer disease-free survival ($p = 0.001$ and 0.03, respectively) (Table 6).

The survival curves for these three biomarkers were done in function of the calculated significant threshold values (2.13 mmol/l, 0.9 nmol/l, and 20 kUI/l, respectively, for HDL-C, testosterone, and CA 15-3) (Figures 2(a), 2(b), 2(e)). For testosterone, two other survival curves were plotted taking into account the hormonotherapy status of patients (Figures 2(c), 2(d)). These latter showed that testosterone was relevant for disease-free survival only in patients treated with hormonotherapy ($p = 0.012$ vs. $p = 0.69$, respectively, for patients with and without hormonotherapy). Using the Cox model, the link between these variables and disease-free survival was tested and demonstrated that only the highest testosterone values predicted increased recurrence risk (HR [CI–95%] = 5.06 [1.66–15.41], $p = 0.004$) (Figure 2(f)).

4. Discussion

In the present study, we determined the effects of PACThe intervention (i.e., medical, nutritional, and psychological monitoring; physical activity training; SPA; and aesthetic care) on the biological and anthropometric status of patients at allocation and after one-year follow-up.

As obesity has an impact on biological status and is a risk factor for breast cancer, we chose to discuss the data according to three BMI subgroups defined as follows:

≤25 kg/m^2 for normal BMI, [25–30 kg/m^2] for overweight, and >30 for obesity. At allocation, the study population's repartition into BMI subgroups was similar to that of the same-age female French population, as previously described [17]. The diet intakes are in accordance with the adult nutritional recommendations for all groups. We noted no difference between the three subgroups but a great variation in declared intakes, particularly in the obese group, raising doubts as to the reliability of the consumption-data collection based on a 72-h self-report.

At allocation, after the completion of breast cancer treatment, the biological and body parameters of the population were in accordance with the usual observed values for normal, overweight, and obesity status. Considering the mean value for each parameter defined as EGIR metabolic syndrome criteria (glucose > 6.1 mmol/l, HDL-C < 1 mmol/l, insulin >18 mUI/l (QR4), and waist circumference > 80 cm), neither overweight nor obesity subgroups met the three required criteria [18]. Among these parameters, only the central criterion of obesity (waist circumference) was above the limit value and emerged as the earliest criterion of metabolic syndrome under our conditions. However, considering the large value dispersion of all these parameters, some patients of both overweight and obese groups could present a metabolic syndrome.

Obesity is well-known to be associated with elevated circulating levels of insulin, insulin-like growth factor 1 (IGF-1), leptin, and inflammation [19]. In our study, we observed a significant increase in CRP, insulin, leptin plasma levels, and the ratio leptin/adiponectin in parallel with significantly increased adiposity markers (fat mass, arm, waist, and hip circumferences). As expected, circulating anti-inflammatory adiponectin was decreased, reinforcing the sub-chronic inflammation associated with obesity and related to the risk of recurrence [20]. Surprisingly, no difference was observed for IGF-1 and testosterone plasma contents, contrary to previous observations [8, 13], probably due to the huge variability of individual values. Their plasma concentrations were maintained in the physiological range for the female population of corresponding age [21, 22].

TABLE 4: Variation in diet and body parameters between one-year follow-up and allocation.

		SPA arm (n = 55)			CTR arm (n = 49)			p value effect of		
		≤25 (n = 29)	]25–30] (n = 15)	>30 (n = 11)	≤25 (n = 23)	25–30 (n = 19)	>30 (n = 7)	SPA	Time	BMI
Diet parameters										
Total energy intake	kcal/d	−41.7 ± 400.5 (−0.02%)	+25.5 ± 556.4 (+10.8%)	−400.1 ± 527.6 (−18.7%)	−227.9 ± 362.6 (−12.3%)	+165.6 ± 410.4 (+20.1%)	−437.6 ± 955.1 (−10.3%)	0.91	**0.039**	0.15
Protein intake	g/d	−4.6 ± 17.9 (−3.9%)	+3.2 ± 25.5 (+17.5%)	−5.8 ± 19.0 (−3.1%)	−1.78 ± 27.1 (+6.0%)	+5.1 ± 21.4 (+26.1%)	−15.4 ± 45.4 (−1.4%)	0.71	0.24	0.35
Carbohydrate intake	g/d	+8.4 ± 52.2 (+10.1%)	+6.1 ± 63.7 (+16.7%)	−57.9 ± 78.3 (−21.5%)	−28.4 ± 54.8 (−9.8%)	+6.7 ± 51.2 (+7.5%)	−46.7 ± 121.1 (−1.5%)	0.84	0.10	0.38
Lipid intake	g/d	−5.5 ± 26.4 (+0.2%)	−1.3 ± 36.4 (+24.0%)	−12.7 ± 25.2 (−13.8%)	−9.4 ± 19.8 (−7.9%)	+13.0 ± 13.0 (+67.3%)	−24.6 ± 45.4 (−21.1%)	0.89	0.099	0.15
Body parameters										
Body weight	kg	−0.10 ± 2.16 (−0.2%)	+1.47 ± 4.00 (+2.2%)	−0.73 ± 5.76 (−0.3%)	−0.24 ± 2.67 (−0.5%)	+0.26 ± 3.86 (+0.4%)	+0.93 ± 2.41 (+1.1%)	0.56	0.45	$<10^{-7}$
Lean mass (LM)	%	+1.03 ± 3.63 (+1.7%)	−0.25 ± 1.66 (−0.4%)	−0.34 ± 5.85 (−0.0%)	0.00 ± 3.20 (−0.0%)	−0.02 ± 3.14 (+0.1%)	+3.09 ± 7.07 (+6.0%)	0.18	0.85	$<10^{-7}$
Fat mass (FM)	%	−0.85 ± 3.50 (−2.7%)	+0.25 ± 1.66 (+0.6%)	+0.31 ± 5.90 (+2.0%)	−0.00 ± 3.20 (0.0%)	+0.02 ± 3.14 (+0.6%)	−1.64 ± 6.05 (−3.6%)	0.11	0.86	$<10^{-7}$
LM/FM ratio		+0.10 ± 0.39 (+5.0%)	−0.01 ± 0.14 (−0.6%)	−0.03 ± 0.37 (+1.6%)	+0.04 ± 0.40 (+1.7%)	−0.01 ± 0.29 (+0.9%)	+0.17 ± 0.39 (+14.4%)	0.20	0.77	$<10^{-7}$
Cell mass	kg	+1.34 ± 6.57 (+6.8%)	+0.95 2.21 (+5.2%)	+0.54 ± 3.02 (+2.0%)	−0.83 ± 4.52 (−2.8%)	−0.66 ± 4.74 (−1.7%)	+2.15 ± 5.82 (+7.3%)	0.19	0.34	$<10^{-7}$
Total water	l	+0.25 ± 1.62 (+0.9%)	+0.51 ± 1.45 (+1.7%)	+0.13 ± 2.53 (+0.8%)	−0.06 ± 1.02 (−0.1%)	+0.11 ± 1.86 (+0.5%)	+1.07 ± 5.15 (+3.2%)	0.22	0.40	$<10^{-7}$
Extracellular water	%	+0.51 ± 1.72 (+2.0%)	−0.67 ± 1.67 (−2.3%)	+0.02 ± 2.33 (+0.6%)	+1.14 ± 4.1 (+4.9%)	0.98 ± 3.72 (+4.3%)	−3.97 ± 10.72 (−7.2%)	0.80	0.86	$<10^{-7}$
Intracellular water	%	+2.21 ± 9.32 (+7.7%)	+0.61 ± 1.32 (+3.2%)	−0.08 ± 1.99 (−0.2%)	−0.35 ± 4.76 (−1.3%)	−0.59 ± 4.68 (−1.9%)	+1.65 ± 4.55 (+7.0%)	0.28	0.53	**0.00012**
Tricipital fold thickness	cm	+0.46 ± 3.74 (+10.7%)	−0.57 ± 7.78 (+1.5%)	−3.25 ± 5.41 (−7.5%)	+0.23 ± 5.05 (+14.1%)	+2.98 ± 5.60 (+25.3%)	−2.51 ± 4.95 (−6.3%)	0.36	0.69	$<10^{-7}$
Arm circumference	cm	−0.10 ± 1.77 (−0.3%)	−1.00 ± 1.49 (−3.1%)	−1.46 ± 1.94 (−3.6%)	−0.46 ± 1.71 (−1.7%)	−0.32 ± 2.16 (−1.1%)	−0.93 ± 2.01 (−2.4%)	0.58	0.32	$<10^{-7}$
Waist circumference (WC)	cm	−2.93 ± 4.41 (−3.7%)	+0.43 ± 3.68 (−0.4%)	−1.09 ± 5.00 (−1.0%)	−0.46 ± 5.65 (−0.4%)	+1.45 ± 8.51 (+2.7%)	−3.36 ± 6.24 (−3.0%)	0.73	0.81	$<10^{-7}$
Hip circumference (HC)	cm	−0.59 ± 2.80 (−0.6%)	1.77 ± 2.83 (+1.7%)	0.23 ± 5.85 (+0.4%)	−1.30 ± 3.56 (−1.1%)	−0.13 ± 3.71 (−0.1%)	2.79 ± 5.53 (2.5%)	0.80	0.66	$<10^{-7}$
WC/HC ratio		−0.03 ± 00.05 (−2.9%)	−0.01 ± 0.02 (−1.5%)	−0.01 ± 0.07 (−1.1%)	+0.01 ± 0.05 (+0.9%)	+0.2 ± 0.08 (+3.1%)	−0.05 ± 0.06 (−4.9%)	0.66	0.61	$<10^{-7}$

Variation for each parameter is expressed in raw value (one-year follow-up value *minus* allocation value) and in percentage of the allocation value: + sign indicates an increase and − sign indicates a decrease. Two-way ANOVA was used to compare longitudinal variations between allocation arms (SPA effect), or one-year follow-up (time effect), or BMI groups (BMI effect), but without interaction test because of unequal class sizes. All tests were two-sided, and the nominal level of significance was 5%. Significant p values are indicated in bold.

Table 5: Variation in biological parameters between one-year follow-up and allocation.

		SPA arm (n = 55)			CTR arm (n = 49)			p value effect of		
		≤25 (n = 29)	25–30 (n = 15)	>30 (n = 11)	≤25 (n = 23)	25–30 (n = 19)	>30 (n = 7)	SPA	Time	BMI
Glucose	mmol/l	0.007 ± 0.446 (0.6%)	0.459 ± 0.748 (8.9%)	0.749 ± 1.358 (15.9%)	−0.089 ± 0.326 (−1.6%)	0.226 ± 0.394 (4.4%)	1.75 ± 2.775 (25.9%)	0.23	**0.04**	**<10^{-6}**
HDL-cholesterol	mmol/l	−0.438 ± 1.471 (−5.6%)	−0.241 ± 1.34 (3.4%)	−0.542 ± 1.31 (−13%)	−0.436 ± 1.384 (−4.8%)	−0.333 ± 1.635 (7%)	−0.028 ± 0.198 (−1.3%)	0.41	**0.027**	**0.007**
Transthyretin	g/l	0.001 ± 0.038 (1%)	0.009 ± 0.026 (3.5%)	−0.005 ± 0.027 (−1.5%)	0.001 ± 0.033 (0.8%)	−0.007 ± 0.031 (−2%)	0.002 ± 0.051 (3.2%)	**0.041**	0.75	0.79
C-reactive protein	mg/l	−0.146 ± 1.142 (11.1%)	0.238 ± 2.818 (27.3%)	−0.127 ± 1.481 (8.9%)	0.264 ± 1.168 (41.8%)	−0.135 ± 4.736 (41.1%)	−1 ± 5.545 (−6.6%)	0.11	0.73	**<10^{-7}**
Insulin	mUI/l	0.17 ± 3.79 (25.7%)	1.97 ± 4.59 (115.7%)	2.51 ± 10.19 (58.4%)	0.22 ± 6.58 (36.1%)	4.47 ± 10.51 (50.9%)	4.53 ± 6.24 (78.4%)	0.41	**0.035**	**<10^{-7}**
IGF-1	μg/l	−0.79 ± 27.51 (6.3%)	−26.61 ± 27.25 (−19.7%)	−18.78 ± 23.9 (−24%)	−13.18 ± 39.9 (−4.2%)	−12.41 ± 34.83 (−12.2%)	9.13 ± 16.55 (16.5%)	0.32	0.072	0.31
Leptin	μg/l	−0.03 ± 1.57 (15.6%)	−0.02 ± 2.72 (9%)	−2.42 ± 6.24 (−2.4%)	0.12 ± 2.38 (21.7%)	1.64 ± 6.28 (23.6%)	−0.93 ± 3.78 (−7.4%)	0.66	0.81	**<10^{-7}**
Adiponectin	mg/l	2.33 ± 4.19 (32.6%)	1.29 ± 2.3 (17.3%)	0.57 ± 1.3 (18%)	0.99 ± 2.51 (13.9%)	1.15 ± 2.65 (11.5%)	0.65 ± 1.01 (5.6%)	0.33	0.082	**0.022**
Leptin/adiponectin ratio		−0.1 ± 0.27 (−2.9%)	−0.08 ± 0.52 (−0.4%)	−1.68 ± 2.92 (−18%)	0.13 ± 1.21 (24.2%)	1.2 ± 4.07 (16.6%)	−0.27 ± 0.29 (−13.6%)	0.73	0.91	**2×10^{-6}**
Testosterone	nmol/l	−0.033 ± 0.299 (−3.6%)	−0.013 ± 0.098 (5%)	0.045 ± 0.347 (10.7%)	−0.051 ± 0.244 (−5%)	0.015 ± 0.332 (3.4%)	0.029 ± 0.757 (21.8%)	0.086	0.83	0.27
CA 15-3	kU/l	2.32 ± 3.08 (18.2%)	0.62 ± 1.78 (6.8%)	0.27 ± 1.71 (4.7%)	−5.32 ± 28.45 (1.4%)	1.56 ± 2.06 (12.5%)	2.71 ± 1.48 (14%)	**0.04**	0.68	**0.07**

Variation for each parameter is expressed in raw value (one-year follow-up value *minus* allocation value) and in percentage of the allocation value: + sign indicates an increase and − sign indicates a decrease. Two-way ANOVA was used to compare longitudinal variations between allocation arms (SPA effect), or one-year follow-up (time effect), or BMI groups (BMI effect), but without interaction test because of unequal class sizes. All tests were two-sided, and the nominal level of significance was 5%. Significant p values are indicated in bold.

TABLE 6: Prognostic value of biological parameters on disease-free survival over 7 years.

Parameters at allocation ($n = 111$)	Median (quartiles)	Threshold		
		$\leq 1^{st}$ quartile	$\leq$ Median	$\leq 3^{rd}$ quartile
Cholesterol-HDL (mmol/l)	1.78 [1.46–2.13]	$p = 0.64$	$p = 0.22$	$p = 0.047^{(+)}$
Testosterone (nmol/l)	0.7 [0.7–0.9]	ND	$p = 0.049^{(-)}$	$p = 0.001^{(-)}$
CA 15-3 (kU/l)	14 [10–20]	$p = 0.28$	$p = 0.07^{(-)}$	$p = 0.03^{(-)}$

Association of biological parameters at allocation with the recurrence risk was tested using a two-sided chi^2 test. The nominal level of significance was 5%. + sign indicates that high values are in favour of a better prognosis, while – sign indicates that these high values worsen prognosis.
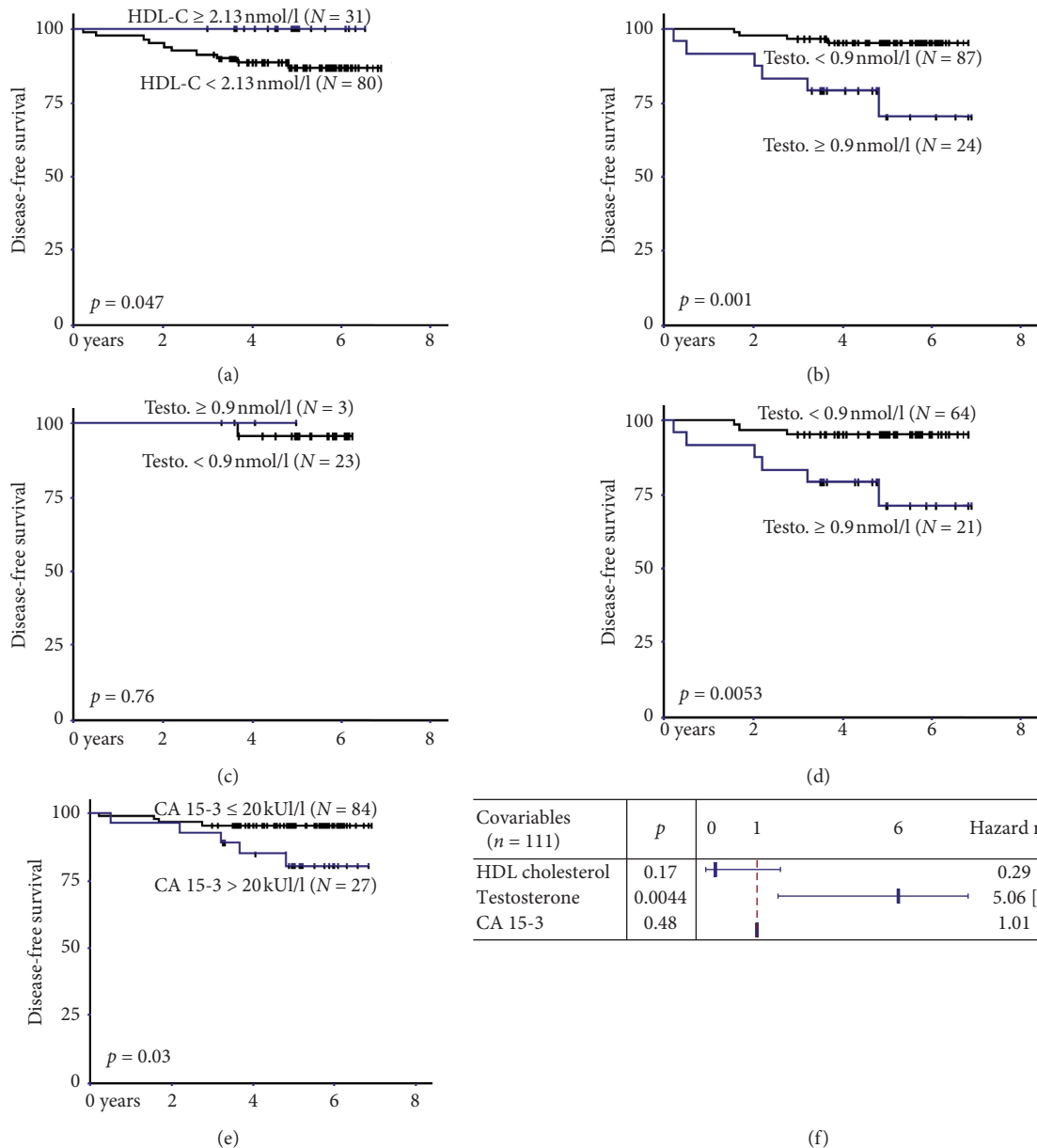
FIGURE 2: Survival curves and hazard ratios for HDL-cholesterol, testosterone, and CA 15-3. (a) HDL-cholesterol. (b) Testosterone—all patients. (c) Testosterone—patients without hormonotherapy. (d) Testosterone—patients with hormonotherapy. (e) CA 15-3. (f) Hazard ratios (Cox model). Threshold values for HDL-cholesterol, testosterone, and CA 15-3 at allocation correspond to the 75% percentile values. They were used to draw survival curves using Kaplan-Meier's method. Comparison of curves was performed using the Log-rank test. Backward stepwise Cox proportional hazard regression model was used to perform the multivariate analysis of survival. All tests were two-sided, and the nominal level of significance was 5%.

Globally, as measurements were performed after completion of breast cancer treatment, body and biological parameters seemed to be more linked to BMI status than to breast disease. Nevertheless, as previously described [23–25], we cannot exclude that the breast cancer therapy may be another cause of metabolic disturbances at allocation. That may be the reason for the great variability observed for all parameters regardless of the BMI subgroup.

One year after inclusion, the impact of the SPA intervention on diet, body, and biological parameters was evaluated. Only transthyretin and CA 15-3 plasma levels were significantly affected by the SPA intervention. Transthyretin, one of the thyroid hormone carriers, is recognized as an acute malnutrition marker whose hepatic synthesis is reduced in case of inflammation [26]. In our study, transthyretin levels remained in the normal range and seemed to be without biological meaning in regard of their tiny variations and the absence of inflammation and of lean mass changes. Breast cancer is generally not associated with malnutrition or sarcopenia, especially so long after treatment [27]. CA 15-3 is frequently used for diagnosis and follow-up of breast cancer [28]. In our study, an *a posteriori* bias appeared for these biomarker data because the CTR group patients presented higher CA 15-3 concentrations than the SPA group at allocation (Supplementary Table 2). One year after treatment completion, as none of the patients was in recurrence, CA 15-3 values decreased under the threshold of 30 kU/l, confirming the efficacy of the therapy [29, 30]. In accordance with previous studies showing modest effects on body and biological parameters of physical activity and nutritional interventions [31, 32], our study shows the lack of one-year impact of a 2-week SPA intervention.

Some metabolic disorder changes were pointed out at one-year follow-up (time effect). Despite a decrease in total energy intake, patients presented an increase in glucose and insulin plasma levels associated with a decrease in HDL-C. These parameters suggest the development of insulin resistance independently of the BMI effect for overweight patients and the reinforcement of insulin resistance for obese patients. These observations are in agreement with previous studies which considered breast cancer as a metabolic disease, with insulin resistance, sub-chronic inflammation, and dysmetabolism induced by therapy [33, 34]. Moreover, an increased risk for metabolic syndrome and obesity has been described in long-term breast cancer survivors [35].

If women with breast cancer frequently lose weight during chemotherapy, a common unwanted long-term effect of this therapy is weight gain, which often ranges 2–6 kg [10, 36] and penalizes mainly patients with adjuvant therapy [37]. In our study, weight gain was modest (less than 1 kg) and concerned mainly the overweight BMI groups, of whom the majority were under hormonal adjuvant therapy. Thus, weight control and diet intervention are important to improve care and control of recurrence risk in posttreatment breast cancer patients [38]. In our study, the reduction in the total energy intake provided by diet modification, especially carbohydrate and lipid intakes, demonstrated the efficacy of patient's nutritional information.

As described at allocation, BMI was the major factor conditioning body and biological parameter changes one year later. For body parameters, we noted high central adiposity (waist and hip circumferences) in the overweight and obese groups. The same biomarker variations were observed and reinforced for the overweight and obese subgroups (i.e., increase in insulin, leptin, and CRP, and decrease in HDL-C). Moreover, these metabolic disorders induced an increased glycaemia and a decreased adiponectinemia in relation to more pronounced insulin resistance and sub-chronic inflammation [20]. Thus, the obese groups presented two EGIR criteria for metabolic syndrome (glucose and waist circumference) one year after breast cancer treatment completion. This confirms previous studies establishing that breast cancer posttreatment increases the risk of metabolic syndrome [39, 40].

Finally, we clarified the link between biological markers at allocation and disease-free survival over seven years of follow-up after breast cancer treatment completion. We confirmed the interest of three biomarkers commonly used in the determination of recurrence risk: the highest plasma values of HDL-C and the lowest plasma values of testosterone and CA 15-3 were associated with a reduced risk of recurrence [41–43]. HDL-C is linked to metabolic disorders and is often related to androgen metabolism [44]. Cholesterol is clearly demonstrated to be a key regulator of breast cancer tumours [45]. Favouring liver cholesterol clearance, an increase in HDL-C limits the availability of cholesterol for recurrent cancer stem cells [46]. In our study, patients with the highest circulating HDL-C presented the lowest recurrence risk. However, this protective effect was not retrieved in the multivariate Cox model, limiting the interest of circulating HDL-C determination in recurrence monitoring.

As previously noted, CA 15-3 is a useful marker for breast cancer follow-up: the circulating value is directly related to the stage and mass of the tumour [29]. In our study, although the lowest circulating CA 15-3 values were associated with the lowest recurrence risk, the multivariate Cox model did not confirm this observation. This is in agreement with the literature, which has established the interest in CA 15-3 for monitoring breast tumour growth, but its poor prognostic value for recurrence risk [28, 30].

In our study, only testosterone presented a significant hazard ratio with disease-free survival; that is, the highest circulating values (>0.9 nmol/l) were associated with recurrence risk multiplied by ≈5 (HR = 5.06 [1.66–15.41]). Notably, this link between testosterone and recurrence risk only applied to patients receiving adjuvant hormonotherapy. This observation confirms Venturelli's observation of increased recurrence risk for testosterone plasma concentration above 0.96 nmol/l with a hazard ratio of 4.68 for overweight women but not for obese ones [47]. Testosterone is strongly associated with the androgen hypothesis of breast carcinogenesis, related to the conversion of androgen into oestrogen by aromatase [13]. This enzymatic activity is

increased in obese patients due to the expansion of adiposity [48]. However, it is not clear whether testosterone *per se* is directly responsible for promoting breast cancer risk or whether it is just a marker of the dysmetabolism linked to overweight and obesity [49]. This later hypothesis was confirmed in our study by the significant correlation of plasma testosterone with several body and biological markers associated with this dysmetabolism (positively with body weight and ratio of WC/HC, and negatively with HDL-C).

Our trial suffers from several limitations:

(1) First, the small numbers of patients divided into different BMI subgroups limited the reliability of the statistical analysis.

(2) Second, the determination of biological parameters at one-year follow-up did not permit the characterization of the short-term benefits of our 2-week SPA intervention. Moreover, the one-year time window could explain the weak impact of this intervention on the biological parameters.

(3) Third, the mismatches observed between diet consumption and weight changes of patients question the reliability of data collection using the 72-h self-reported diet questionnaire.

Few studies investigating the benefits of physical activity and nutritional interventions in cancer survivors have considered the biological status of the patients in their outcomes. Our data demonstrated that the health changes of patients were mainly related to their body condition and highlighted the importance of evaluating biological and anthropometric status in monitoring cancer survivors.

5. Conclusion

To conclude, our study shows that one year after a global multidisciplinary supportive and educational intervention, few anthropometric and biological changes could be attributed to this intervention. It demonstrates that the one-year changes of patients are mainly related to their body mass index (BMI) and confirms the importance of taking into account biological markers of metabolic status in the follow-up of posttherapy breast disease. Among the tools needed for this monitoring, our study highlights the interest of plasma testosterone in the evaluation of recurrence risk. These observations may help reinforce care recommendations for cancer survivors but need to be confirmed on a large population for a more comprehensive approach. Future studies would permit a better understanding of the mechanisms by which such multidisciplinary interventions could interact with breast cancer recurrence and help define the most effective modalities.

Data Availability

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

Additional Points

Highlights. (1) After breast cancer treatment completion, changes in anthropometric and biological parameters are mainly dependent on the patient's BMI level. (2) A reinforcement of insulin resistance is observed in overweight and obese patients after one-year treatment completion, independently of physical activity and nutritional intervention. (3) Testosterone plasma levels at the time of treatment completion are associated with recurrence risk in patients receiving adjuvant hormonotherapy.

Ethical Approval

The protocol was approved by the AFSSAPS (French Agency for Sanitary Security of Health Products), the regional Ethics Committee (2008), and the French National Committee controlling personal computerized data (CNIL). This trial was performed in compliance with the Helsinki declaration and registered in ClinicalTrials.gov with the no. NCT01563588.

Consent

Written informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

M.-P. V., F. K., M. D., and Y.-J. B. contributed in study conception and design. M.-A. M.-R., I. V. P.-D., A. T., and S. J. contributed in patient inclusion and follow-up. M.-P. V., F. K., and A. R. contributed in acquisition, analysis, and interpretation of the data. M.-P. V., F. K., A. R., and Y.-J. B. drafted the manuscript. All authors gave the final approval.

Supplementary Materials

Table 1: diet, body, and biological parameters for the SPA arm at allocation and one-year follow-up. Table 2: diet, body, and biological parameters for the CTR arm at allocation and

one-year follow-up. Overall protocol design. (*Supplementary Materials*)

References

[1] G. Gresham, J. Schrack, L. M. Gresham et al., "Wearable activity monitors in oncology trials: current use of an emerging technology," *Contemporary Clinical Trials*, vol. 64, pp. 13–21, 2018.

[2] J. A. Ligibel and H. D. Strickler, "Obesity and its impact on breast cancer: tumor incidence, recurrence, survival, and possible interventions," *American Society of Clinical Oncology Educational Book*, vol. 33, pp. 52–59, 2013.

[3] K. E. Post and J. Flanagan, "Web based survivorship interventions for women with breast cancer: an integrative review," *European Journal of Oncology Nursing*, vol. 25, pp. 90–99, 2016.

[4] S. Y. Loh and A. Nadia, "Methods to improve rehabilitation of patients following breast cancer surgery: a review of systematic reviews," *Breast Cancer: Targets and Therapy*, vol. 2015, no. 7, pp. 81–98, 2015.

[5] S. I. Mishra, R. W. Scherer, P. M Geigle et al., "Exercise interventions on health-related quality of life for cancer survivors," *The Cochrane Database of Systematic Reviews*, vol. 15, no. 8, Article ID CD007566, 2012.

[6] M. C. De Boer, E. A. Wörner, D. Verlaan, and P. A. M. Van Leeuwen, "The mechanisms and effects of physical activity on breast cancer," *Clinical Breast Cancer*, vol. 17, no. 4, pp. 272–278, 2017.

[7] G. Secreto, E. Venturelli, E. Meneghini et al., "Testosterone and biological characteristics of breast cancers in postmenopausal women," *Cancer Epidemiology Biomarkers & Prevention*, vol. 18, no. 11, pp. 2942–2948, 2009.

[8] L. Mauro, G. D. Naimo, E. Ricchio, M. L. Panno, and S. Andò, "Cross-talk between adiponectin and IGF-IR in breast cancer," *Frontiers in Oncology*, vol. 5, p. 157, 2015.

[9] N. M. Iyengar, X. K. Zhou, A. Gucalp et al., "Systemic correlates of white adipose tissue inflammation in early-stage breast cancer," *Clinical Cancer Research*, vol. 22, no. 9, pp. 2283–2289, 2016.

[10] M. N. Kirshbaum, "A review of the benefits of whole body exercise during and after treatment for breast cancer," *Journal of Clinical Nursing*, vol. 16, no. 1, pp. 104–121, 2007.

[11] L. L. Peterson and J. A. Ligibel, "Physical activity and breast cancer: an opportunity to improve outcomes," *Current Oncology Reports*, vol. 20, no. 7, p. 50, 2018.

[12] C. L. Rock, C. Pande, S. W. Flatt et al., "Favorable changes in serum estrogens and other biologic factors after weight loss in breast cancer survivors who are overweight or obese," *Clinical Breast Cancer*, vol. 13, no. 3, pp. 188–195, 2013.

[13] D. Coradini, A. Orenti, E. Venturelli, A. Cavalleri, E. Biganzoli, and S. Oriana, "Serum levels of testosterone and SHBG in association with body mass index improve the predictive capability of consolidate tumor biomarkers in pre- and postmenopausal breast cancer patients," *Japanese Journal of Clinical Oncology*, vol. 48, no. 4, pp. 308–316, 2018.

[14] F. Kwiatkowski, M. A. Mouret-Reynier, M. Duclos et al., "Long term improved quality of life by a 2-week group physical and educational intervention shortly after breast cancer chemotherapy completion. Results of the 'programme of accompanying women after breast cancer treatment completion in thermal resorts' (PACThe) randomised clinical trial of 251 patients," *European Journal of Cancer*, vol. 49, no. 7, pp. 1530–1538, 2013.

[15] F. Kwiatkowski, M.-A. Mouret-Reynier, M. Duclos et al., "Long-term improvement of breast cancer survivors' quality of life by a 2-week group physical and educational intervention: 5-year update of the 'PACThe' trial," *British Journal of Cancer*, vol. 116, no. 11, pp. 1389–1393, 2017.

[16] F. Kwiatkowski, M. Girard, K. Hacene, and J. Berlie, "Sem: a suitable statistical software adaptated for research in oncology," *Bull Cancer*, vol. 87, no. 10, pp. 715–721, 2000.

[17] M. Arnold, M. Touillaud, L. Dossus et al., "Cancers in France in 2015 attributable to high body mass index," *Cancer Epidemiology*, vol. 52, pp. 15–19, 2018.

[18] B. Balkau and M. A. Charles, "Comment on the provisional report from the WHO consultation. European group for the study of insulin resistance (EGIR)," *Diabet Medicine*, vol. 16, no. 5, pp. 442-443, 1999.

[19] D. F. Argolo, C. A. Hudis, and N. M. Iyengar, "The impact of obesity on breast cancer," *Current Oncology Reports*, vol. 20, no. 6, p. 47, 2018.

[20] S.-A. Lee, H. Sung, W. Han, D.-Y. Noh, S.-H. Ahn, and D. Kang, "Serum adiponectin but not leptin at diagnosis as a predictor of breast cancer survival," *Asian Pacific Journal of Cancer Prevention*, vol. 15, no. 15, pp. 6137–6143, 2014.

[21] H. Ketha and R. J. Singh, "Clinical assays for quantitation of insulin-like-growth-factor-1 (IGF1)," *Methods*, vol. 81, pp. 93–98, 2015.

[22] K. M. Wooding, J. A. Hankin, C. A. Johnson et al., "Measurement of estradiol, estrone, and testosterone in postmenopausal human serum by isotope dilution liquid chromatography tandem mass spectrometry without derivatization," *Steroids*, vol. 96, pp. 89–94, 2015.

[23] T. Coskun, F. Kosova, Z. Ari, A. Sakarya, and Y. Kaya, "Effect of oncological treatment on serum adipocytokine levels in patients with stage II-III breast cancer," *Molecular and Clinical Oncology*, vol. 4, no. 5, pp. 893–897, 2016.

[24] A. Alacacioglu, L. Kebapcilar, Z. Gokgoz et al., "Leptin, insulin and body composition changes during adjuvant taxane based chemotherapy in patients with breast cancer, preliminary study," *Indian Journal of Cancer*, vol. 53, no. 1, pp. 39–42, 2016.

[25] J. Bao, N. Borja, M. Rao et al., "Impact of weight change during neoadjuvant chemotherapy on pathologic response in triple-negative breast cancer," *Cancer Medicine*, vol. 4, no. 4, pp. 500–506, 2015.

[26] A. Tsuboi, M. Terazawa-Watanabe, T. Kazumi, and K. Fukuo, "Associations of decreased serum transthyretin with elevated high-sensitivity CRP, serum copper and decreased hemoglobin in ambulatory elderly women," *Asia Pacific Journal of Clinical Nutrition*, vol. 24, no. 1, pp. 83–89, 2015.

[27] S. Steinbach, T. Hummel, C. Böhner et al., "Qualitative and quantitative assessment of taste and smell changes in patients undergoing chemotherapy for breast cancer or gynecologic malignancies," *Journal of Clinical Oncology*, vol. 27, no. 11, pp. 1899–1905, 2009.

[28] J. G. Santillán-Benítez, H. Mendieta-Zerón, L. M. Gómez-Oliván et al., "The tetrad BMI, leptin, leptin/adiponectin (L/A) ratio and CA 15-3 are reliable biomarkers of breast cancer," *Journal of Clinical Laboratory Analysis*, vol. 27, no. 1, pp. 12–20, 2013.

[29] Y. Fujimoto, T. Higuchi, A. Nishimukai et al., "High levels of serum CA15-3 and residual invasive tumor size are associated with poor prognosis for breast cancer patients with non-pathological complete response after neoadjuvant chemotherapy," *Journal of Surgical Oncology*, vol. 118, no. 1, pp. 228–237, 2018.

[30] A. Nicolini, A. Carpi, P. Ferrari et al., "An individual reference limit of the serum CEA-TPA-CA 15-3 tumor marker panel in the surveillance of asymptomatic women following surgery for primary breast cancer," *Cancer Management and Research*, vol. 10, pp. 6879–6886, 2018.

[31] R. T. Chlebowski, "Nutrition and physical activity influence on breast cancer incidence and outcome," *The Breast*, vol. 22, no. Suppl 2, pp. S30–S37, 2013.

[32] A.-M. Foucaut, S. E. Berthouze-Aranda, M. Touillaud et al., "Reduction of health risk factors through an adapted physical activity program in patients with breast cancer," *Supportive Care in Cancer*, vol. 22, no. 4, pp. 1097–1104, 2014.

[33] R. Bhandari, G. A. Kelley, T. A. Hartley, and I. R. H. Rockett, "Metabolic syndrome is associated with increased breast cancer risk: a systematic review with meta-analysis," *International Journal of Breast Cancer*, vol. 2014, Article ID 189384, 13 pages, 2014.

[34] D. T. Dibaba, D. Braithwaite, and T. Akinyemiju, "Metabolic syndrome and the risk of breast cancer and subtypes by race, menopause and BMI," *Cancers*, vol. 10, no. 9, p. 299, 2018.

[35] A. Engin, "Obesity-associated breast cancer: analysis of risk factors," in *Obesity and Lipotoxicity*, vol. 960, pp. 571–606, Springer, Berlin, Germany, 2017.

[36] M. Sadim, Y. Xu, K. Selig et al., "A prospective evaluation of clinical and genetic predictors of weight changes in breast cancer survivors," *Cancer*, vol. 123, no. 13, pp. 2413–2421, 2017.

[37] A. Ginzac, É. Thivat, M.-A. Mouret-Reynier et al., "Weight evolution during endocrine therapy for breast cancer in postmenopausal patients: effect of initial fat mass percentage and previous adjuvant treatments," *Clinical Breast Cancer*, vol. 18, no. 5, pp. e1093–e1102, 2018.

[38] M. De Roon, A. M. May, A. McTiernan et al., "Effect of exercise and/or reduced calorie dietary interventions on breast cancer-related endogenous sex hormones in healthy postmenopausal women," *Breast Cancer Research*, vol. 20, no. 1, p. 81, 2018.

[39] W. K. Cho, D. H. Choi, W. Park et al., "Effect of body mass index on survival in breast cancer patients according to subtype, metabolic syndrome, and treatment," *Clinical Breast Cancer*, vol. 18, no. 5, pp. e1141–e1147, 2018.

[40] V. Boonyaratanakornkit and P. Pateetin, "The role of ovarian sex steroids in metabolic homeostasis, obesity, and postmenopausal breast cancer: molecular mechanisms and therapeutic implications," *BioMed Research International*, vol. 2015, Article ID 140196, 13 pages, 2015.

[41] E. R. Nelson, "The significance of cholesterol and its metabolite, 27-hydroxycholesterol in breast cancer," *Molecular and Cellular Endocrinology*, vol. 466, pp. 73–80, 2018.

[42] A. Micheli, E. Meneghini, G. Secreto et al., "Plasma testosterone and prognosis of postmenopausal breast cancer patients," *Journal of Clinical Oncology*, vol. 25, no. 19, pp. 2685–2690, 2007.

[43] P. Kachhawa, K. Kachhawa, D. Agrawal, V. Sinha, P. D. Sarkar, and S. Kumar, "Association of dyslipidemia, increased insulin resistance, and serum CA 15-3 with increased risk of breast cancer in urban areas of north and central India," *Journal of Mid-Life Health*, vol. 9, no. 2, pp. 85–91, 2018.

[44] M. Voisin, P. De Medina, A. Mallinger et al., "Identification of a tumor-promoter cholesterol metabolite in human breast cancers acting through the glucocorticoid receptor," *Proceedings of the National Academy of Sciences*, vol. 114, no. 44, pp. E9346–E9355, 2017.

[45] C. Bobin-Dubigeon, A. Chauvin, V. Brillaud-Meflah, F. Boiffard, M.-P. Joalland, and J.-M. Bard, "Liver X receptor (LXR)-regulated genes of cholesterol trafficking and breast cancer severity," *Anticancer Research*, vol. 37, no. 10, pp. 5495–5498, 2017.

[46] K. Pelton, C. M. Coticchia, A. S. Curatolo et al., "Hypercholesterolemia induces angiogenesis and accelerates growth of breast tumors *in vivo*," *The American Journal of Pathology*, vol. 184, no. 7, pp. 2099–2110, 2014.

[47] E. Venturelli, A. Orenti, A. S. C. Fabricio et al., "Observational study on the prognostic value of testosterone and adiposity in postmenopausal estrogen receptor positive breast cancer patients," *BMC Cancer*, vol. 18, no. 1, p. 651, 2018.

[48] C. Gérard and K. A. Brown, "Obesity and breast cancer—role of estrogens and the molecular underpinnings of aromatase regulation in breast adipose tissue," *Molecular and Cellular Endocrinology*, vol. 466, pp. 15–30, 2018.

[49] F. Bertolini, "Adipose tissue and breast cancer progression: a link between metabolism and cancer," *The Breast*, vol. 22, pp. S48–S49, 2013.

Research Article

Dual HER2 Blockade versus a Single Agent in Trastuzumab-Containing Regimens for HER2-Positive Early Breast Cancer: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

Liuwen Yu,[1] Fangmeng Fu ![ORCID],[1] Jing Li,[1] Meng Huang,[2] Bangwei Zeng,[3] Yuxiang Lin,[1] Qian Mei,[1] Jinxing Lv,[1] and Chuan Wang ![ORCID][1]

[1]*Breast Surgery Ward, Department of General Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian Province, China*
[2]*Fujian Center for Disease Control and Prevention, Fuzhou, Fujian Province, China*
[3]*Nosocomial Infection Control Branch, Fujian Medical University Union Hospital, Fuzhou, Fujian Province, China*

Correspondence should be addressed to Fangmeng Fu; fufangmengqz@hotmail.com and Chuan Wang; chuanwang1968@outlook.com

Purpose. Although trastuzumab is the standard of care for patients with human epidermal growth factor receptor 2 (HER2)- positive early breast cancer (EBC), drug resistance and disease relapse occur. Therefore, we performed a meta-analysis to assess the efficacy and safety of trastuzumab-containing dual anti-HER2 therapy compared to trastuzumab alone. *Methods*. A systematic search was performed to identify eligible randomized controlled trials (RCTs). Main outcomes including event-free survival/invasive disease-free survival (EFS/iDFS), overall survival (OS), and safety were considered. *Results*. Ten RCTs were included (15,284 patients). Significant improvements were observed in both EFS/iDFS (HR 0.86, $p = 0.0003$) and OS (HR 0.86, $p = 0.02$) with trastuzumab-based dual anti-HER2 therapy, especially in adjuvant treatment, while in the neoadjuvant setting, dual-targeted therapy also achieved a substantial pathological complete response (pCR) benefit (HR 1.34, $p = 0.0002$). Subgroup analysis revealed that the EFS/iDFS benefit was slightly higher with trastuzumab plus pertuzumab or plus neratinib than trastuzumab plus lapatinib, while OS benefit was significant with trastuzumab plus lapatinib, but there were no subgroup differences (interaction test, $p = 0.80$ and 0.24, resp.). In addition, EFS/iDFS benefit was unrelated to hormone receptor status but pronounced in the lymph node-positive (LN+) subgroup, which should be interpreted cautiously for lacking interaction ($p = 0.18$). Besides, patients receiving dual therapy, especially with the lapatinib-containing regimen, experienced more toxicity, but no increase in cardiotoxicity. *Conclusions*. Despite being associated with more toxicity, trastuzumab-containing dual anti-HER2 therapy is superior to trastuzumab single agent for HER2-positive EBC independent of hormone receptor status. The correlation between survival and LN status needs further verification. Trastuzumab plus pertuzumab or plus neratinib is the preferred regimen with substantial efficacy and lower toxicity.

1. Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer-related deaths in women [1]. It is a heterogeneous disease and divided into four major molecular subtypes based on gene expression [2], of which the human epidermal growth factor receptor 2- (HER2-)

positive subtype accounts for 15%–20% of breast cancers (BC) and is associated with a worse prognosis [3–5].

HER2 belongs to the human epidermal growth factor receptor (EGFR/HER/ErbB) family which also includes HER1 (EGFR), HER3, and HER4. HER receptors are transmembrane glycoproteins that comprise an extracellular ligand-binding region and an intracellular tyrosine kinase

domain [6]. Trastuzumab (Herceptin), a monoclonal antibody against subdomain IV of the HER2extracellular domain (ECD), combined with chemotherapy can significantly improve the prognosis of HER2-positive BC patients compared with chemotherapy alone, which has been demonstrated in the Cochrane meta-analyses [7, 8]. Furthermore, according to several large and long-term followup trials, one year of trastuzumab therapy plus chemotherapy has become the standard of care for HER2-positive early breast cancer (EBC) patients [9–12]. However, cases of drug resistance remain and about 30% of patients relapse after trastuzumab therapy and new approaches are required [10–12].

Following trastuzumab, other HER2-targeting agents including lapatinib [13], pertuzumab [14], and neratinib [15] have been approved by the US Food and Drug Administration (FDA) for the treatment of HER2+ BC. Pertuzumab, another humanized monoclonal antibody, differs from trastuzumab in that it binds to the extracellular domain II of HER2 and inhibits homodimer or heterodimer formation, which has complementary mechanisms of action with trastuzumab to improve the efficacy of cancer therapy [16]. Lapatinib and neratinib are both oral, small molecule tyrosine kinase inhibitors that can further enhance HER2 inhibition by blocking intracellular signaling pathways [17]. The difference is that lapatinib is a dual reversible inhibitor of HER1 and HER2 tyrosine kinases, while neratinib is an irreversible inhibitor of HER1, HER2, and HER4.

Further studies focused on identifying biomarkers that may effectively predict which patients will respond best to HER2-targeted therapies. The I-SPY 2 trial, an adaptive phase 2 trial, identifying eight biomarker subtypes with considering HER2 status, hormone receptor status, and risk based on a 70-gene profile, found that neratinib was more likely to have an increased pathological complete response (pCR) rate than trastuzumab when added to standard chemotherapy in patients with HER2-positive (HER2+) and hormone-receptor-negative (HR−) BC [18]. Veeraraghavan et al. found that a clinical subtype in breast cancer with high HER2 amplification and an intact PI3K pathway has a better response to anti-HER2 therapies without chemotherapy [19]. The findings of Kim et al. showed that discordance between IHC-based subtypes and PAM50-based intrinsic subtypes was related to inadequate treatment and diminished survival in BC [20]. Studies also indicated that the percentage of stromal tumor-infiltrating lymphocytes (TILs) was associated with a higher pCR rate and improved survival in patients with HER2 + BC [21–23]. The optimal predictive biomarkers need further validation to contribute to development of precision medicine.

Clinical studies have shown that combining different anti-HER2 agents with complementary mechanisms may overcome drug resistance and be more effective than singleagent therapy. In the neoadjuvant setting, the NeoSphere trial confirmed dual blockade with trastuzumab plus pertuzumab produced a higher pathological complete response (pCR) which was pronounced in the hormone receptornegative (HR−) patients [24]. The NeoALTTO trial demonstrated trastuzumab plus lapatinib therapy also

significantly improved pCR [25]. In the adjuvant setting, the NCCN Guidelines recommended trastuzumab plus pertuzumab as an option for ≥T2 and ≥N1 HER2-positive patients because the APHINITY trial showed a substantial invasive disease-free survival (iDFS) benefit from trastuzumab plus pertuzumab, especially in lymph node-positive (LN+) patients [26, 27]. However, the ALTTO trial reported no substantial DFS benefit from trastuzumab plus lapatinib therapy and there was higher toxicity [28]. The use of dual anti-HER2 therapy and the most beneficial subgroups of patients as well as the correlated toxicities still needs further exploration.

Thus, we conducted a meta-analysis to evaluate the efficacy and safety of using trastuzumab-containing dual antiHER2 regimens versus standard trastuzumab alone regimen in patients with HER2-positive EBC and to identify the optimal dual anti-HER2 regimens, as well as the subgroup of patients who would most likely benefit from dual therapy.

2. Methods

2.1. Eligibility Criteria. We included prospective phase II/III randomized controlled trials (RCTs) that assessed the efficacy or safety of trastuzumab-containing dual anti-HER2 therapy versus trastuzumab single-agent therapy in patients with HER2-positive EBC. We excluded patients with metastatic BC and studies with insufficient outcomes data.

2.2. Outcome Measures. The primary outcomes were eventfree survival/invasive disease-free survival (EFS/iDFS) and overall survival (OS). The secondary outcomes were overall response rate (ORR), pCR rate in breast and axillary LNs, cardiac toxicity, and other toxicities. For definitions of outcomes, see Additional file 1: Appendix 1.

2.3. Search Strategy. We searched Cochrane Central Register of Controlled Trials (CENTRAL), EMBASE, MEDLINE, and ClinicalTrials.gov for eligible RCTs up to December 2018. We also screened relevant abstracts from the San Antonio Breast Cancer Symposium (SABCS), American Society of Clinical Oncology (ASCO), and European Society for Medical Oncology (ESMO) Meeting as well as related metaanalyses, reviews, and editorials of HER2-positive BC. The following keywords were adopted: breast cancer, trastuzumab (Herceptin), pertuzumab (Perjeta), lapatinib (Tykerb), neratinib (HKI-272), afatinib (BIBW-2992), and MM-111.

2.4. Data Extraction and Quality Assessment. Two authors extracted the data independently and assessed the quality of each trial according to the risk of bias tool of The Cochrane Collaboration [29] and any discrepancies were resolved by consensus or consulting a third author.

2.5. Data Synthesis. We estimated pooled hazard ratios (HRs) for survival outcomes (OS, EFS/iDFS) and risk ratios (RRs) for dichotomous outcomes (ORR, pCR, and toxicities) with 95% confidence intervals (CIs) using the inverse-

variance method of RevMan5.3 software [29]. The random-effects model was adopted to combine heterogeneity across studies.

We used χ^2 and I^2 statistics to quantify heterogeneity. Significant heterogeneity existed if $p < 0.10$ or $I^2 > 50\%$. The following subgroup analyses were performed: treatment setting (neoadjuvant or adjuvant setting), dual anti-HER2 regimen (trastuzumab plus lapatinib, trastuzumab plus pertuzumab, or trastuzumab plus neratinib), chemotherapy regimen (taxane-containing non-anthracycline, anthracycline plus taxane, or others), LN status, and hormone receptor status. We carried out sensitivity analyses for main outcomes and those with substantial heterogeneity using the leave-one-out procedure. The impact of small-study and reporting bias was assessed using funnel plots and Begg's test through Stata/SE 11.2 software [30].

3. Results

3.1. Study Selection and Characteristics. We searched and identified 10 studies corresponding to 16 publications with a total of 15,284 participants for the meta-analysis [24, 28, 31–42]. The flow diagram of study selection is shown in Figure 1. For characteristics of the included studies, see in Table 1. For details, see Additional file 1: Appendix 2.

The median follow-up time varied from 3.8 y to 6.9 y. Seven trials assessed the role of the dual HER2 blockade in a neoadjuvant setting [24, 31–33, 35, 36, 38], while three trials assessed the adjuvant setting [26, 28, 42]. There were seven trials of a trastuzumab plus lapatinib regimen [28, 31–33, 35, 36, 38], two trials considered a trastuzumab plus pertuzumab regimen [24, 26], and one trial considered a trastuzumab plus neratinib regimen [42]. Overall survival of the ExteNET trial was not reported [42]. The "risk of bias" assessment for each trial is shown in the Additional file 1: Appendix 3.

3.2. Effects of Interventions. The forest plots for all outcomes are included in Figure S1 (Additional file 2).

3.2.1. Overall Survival. Four studies reported data about OS for pooling in meta-analyses [26, 28, 31, 36], excluding that by Martin et al. that has not reached the planned 248 events [42]. The pooled OS data demonstrated a statistically significant improvement for patients who received trastuzumab-containing dual anti-HER2 therapy compared to trastuzumab single-agent therapy (HR 0.86, 95% CI 0.75–0.98, $p = 0.02$; Figure 2). There was no heterogeneity across studies ($I^2 = 0\%$, $p = 0.86$).

Subgroup analyses of treatment setting suggested that the survival benefit from the dual HER2 block was on the margins of statistical significance in adjuvant treatment (HR 0.87, 95% CI 0.65–1.00, $p = 0.05$), but no significance in neoadjuvant treatment (HR 0.62, 95% CI 0.35–1.10, $p = 0.10$). No subgroup differences were observed (interaction test, $p = 0.26$). In a subgroup analysis according to type of dual HER2 blockade regimen, the dual therapy with trastuzumab plus lapatinib (HR 0.85, 95% CI 0.73–0.99,

$p = 0.03$) significantly improved the OS compared to trastuzumab plus pertuzumab (HR 0.89, 95% CI 0.66–1.19, $p = 0.42$). However, there were no subgroup differences (interaction test, $p = 0.80$).

3.2.2. Event-Free Survival/Invasive Disease-Free Survival. The EFS/iDFS was reported in 5/10 studies [24, 26, 28, 31, 42]. There was a substantial benefit with dual HER2 blocking (HR 0.86, 95% CI 0.79–0.93, $p = 0.0003$; Figure 3) with no heterogeneity among studies ($I^2 = 0\%$, $p = 0.57$).

Subgroup analyses of treatment setting indicated a substantial EFS/iDFS benefit with dual blockade in an adjuvant setting (HR 0.86, 95% CI 0.78–0.94, $p = 0.001$) versus the neoadjuvant setting (HR 0.75, 95% CI 0.49–1.13, $p = 0.17$), but no subgroup difference (interaction test, $p = 0.52$). In a subgroup analysis according to type of dual anti-HER2 regimen, higher EFS/iDFS benefits were observed in the regimens with trastuzumab plus neratinib (HR 0.73, 95% CI 0.58–0.93, $p = 0.01$; Figure 4) and trastuzumab plus pertuzumab (HR 0.80, 95% CI 0.65–0.98, $p = 0.03$; Figure 4) than trastuzumab plus lapatinib (HR 0.90, 95% CI 0.81–0.99, $p = 0.03$; Figure 4). However, no subgroup differences were found (interaction test, $p = 0.24$).

Furthermore, we also found that the benefit of EFS/iDFS with a dual HER2 block in the LN + subgroup (HR 0.75, 95% CI 0.63–0.88, $p = 0.0005$; Figure 5(a)) was superior to the LN-subgroup (HR 1.01, 95% CI 0.67–1.53, $p = 0.95$; Figure 5(a)) but was not associated with the hormone receptor status (Figure 5(b)). However, the interaction test suggested that the EFS/iDFS benefit does not depend on LN status ($p = 0.18$).

3.2.3. Overall Response Rate. The ORR data from five studies were analyzed [24, 25, 35, 36, 38]. We excluded Guarneri et al. [33] in which the clinical objective response was reported as approximately 90% without further information. The difference in ORR did not reach statistical significance in either the pooled analysis (RR 1.03, 95% CI 0.96–1.10, $p = 0.45$) or the subgroup analysis of the dual anti-HER2 regimen.

3.2.4. Pathological Complete Response. Seven neoadjuvant studies reported pCR data [24, 31–33, 35, 36, 38]. The pCR rates for the dual-targeted group and monotherapy group were 51.60% and 38.26%. There was a significant 13.34% absolute improvement (RR 1.34, 95% CI 1.15–1.57, $p = 0.0002$) with no substantial heterogeneity ($I^2 = 34\%$, $p = 0.17$).

Subgroup analyses of dual anti-HER2 regimens showed a pCR rate favouring the regimen of trastuzumab plus pertuzumab (RR 1.83, 95% CI 1.19–2.81, $p = 0.006$) versus trastuzumab plus lapatinib (RR 1.29, 95% CI 1.12–1.48, $p = 0.0003$). A similar benefit was found in the HR– subgroup (RR 1.29, 95% CI 1.06–1.56, $p = 0.01$) rather in the HR+ subgroup (RR 1.12, 95% CI 0.92–1.37, $p = 0.25$) in the subgroup analysis of hormone receptor status. However,
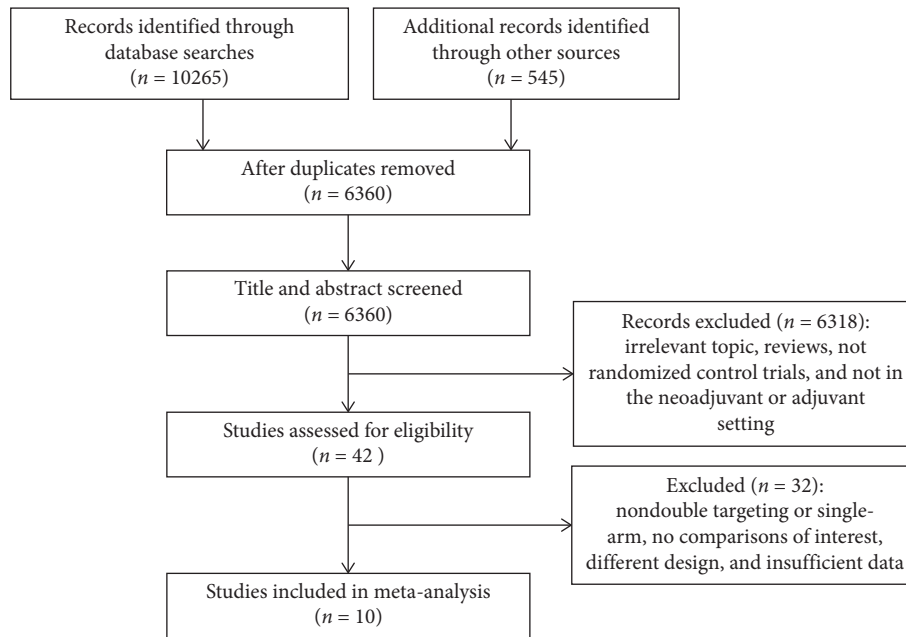
Figure 1: The process diagram of studies search and selection in the meta-analysis.

there were no subgroup differences between pCR and the type of dual anti-HER2 regimens or hormone receptor status (interaction test, $p = 0.13$ and 0.34, resp.).

3.3. Safety.

The forest plots for all outcomes are included in Figure S1 (Additional file 2).

3.3.1. Cardiac Toxicities.

Eight studies assessing cardiotoxicity were pooled in the meta-analysis [24–26, 28, 32, 33, 36, 38]. There was no significant difference in cardiotoxicity between trastuzumab-containing dual-targeting therapy and trastuzumab alone therapy (RR 1.14, 95% CI 0.63–2.05, $p = 0.66$, Figure 6).

In the subgroup analysis of a treatment setting, no significant cardiotoxicity was observed either in the neoadjuvant setting (RR 0.92, $p = 0.88$) or in the adjuvant setting (RR 1.38, $p = 0.51$). Subgroup analysis stratified by congestive heart failure (CHF) and left ventricular ejection fraction (LVEF) decline showed no substantial increase in CHF (RR 0.45, $p = 0.28$) and LVEF decline (RR 0.95, $p = 0.31$) in patients receiving dual-targeting therapy. Moreover, we performed subgroup analyses for CHF and LVEF, stratified by the type of dual anti-HER2 regimen and the type of chemotherapy, and no statistical difference was observed. In our meta-analysis, LVEF decline was defined as reported by the authors of included studies because different thresholds were used. More events in the APHINITY and ALTTO trials may be due to the fact that large enrolled population and broad definition of LVEF decline were used, so we also performed the corresponding analyses using the narrow definition of LVEF decline and the results also showed no significant statistical difference.

3.3.2. Other Toxicities.

We conducted analyses of other common grade 3/4 toxicities reported in more than half of the trials: diarrhea (10 studies), hepatic toxicity (9 studies), skin disorder (9 studies), neutropenia (8 studies), febrile neutropenia (7 studies), nausea and vomiting (5 studies), and fatigue (5 studies).

Patients receiving dual HER2 blocking therapy had a significant increase in the incidence of grade 3/4 diarrhea (RR 8.22, 95% CI 3.89–17.38, $p < 0.00001$), hepatic toxicity (RR 2.32, 95% CI 1.30–4.14, $p = 0.004$), skin disorder (RR 4.20, 95% CI 2.40–7.34, $p < 0.00001$), and nausea and vomiting (RR 3.51, 95% CI 1.19–10.38, $p = 0.02$). There were no statistical differences in the incidence of neutropenia, febrile neutropenia, or fatigue.

Subgroup analysis of dual anti-HER2 regimens and chemotherapy regimens was performed for each toxicity, the results showed that diarrhea was mainly associated with the trastuzumab plus neratinib group and trastuzumab plus lapatinib group, and hepatic toxicity and skin disorders were mainly associated with the trastuzumab plus lapatinib group, while nausea and vomiting were associated with the trastuzumab plus neratinib group. And a taxane-containing non-anthracycline regimen has a lower risk of diarrhea than an anthracycline plus taxane regimen. No other differences were observed in the subgroup analyses.

3.4. Sensitivity Analyses and Publication Bias.

As most of the outcomes did not show significant heterogeneity, we carried out sensitivity analyses for OS, EFS/iDFS, ORR, pCR, and cardiac toxicity and the results were stable (Additional file 2: Figure S2). The funnel plots and Begg's test for OS and EFS/iDFS indicated no evidence of publication bias (Additional file 2: Figure S3).

TABLE 1: Characteristics of the included studies.

Study	Phase	N	MF	Neoadjuvant treatment Chemotherapy (wks)	Neoadjuvant Anti-HER2 therapy	Adjuvant Chemotherapy (wks)	Adjuvant Anti-HER2 therapy	Arms	LN+ patients (%)	HR+ patients (%)	Duration# (wks)	Outcomes
Adjuvant setting												
ExteNET* (Martin1 2017[a]; Martin2 2017[a]; Chan 2016)	III	2840	5.2 y	UNK	T or none	UNK	T + N	1420	1085 (76)	816 (57)	52	DFS, IDFS, OS, and safety
							T	1420	1084 (76)	815 (57)		
ALTTO* (Piccart-Gebhart 2016; Moreno-Aspitia1 2017[b]; Moreno-Aspitia2 2017[b])	III	8381	6.9 y	UNK	None	Design 1: chemotherapy × (12–18) Design 2: A × (9–12) + Taxane × 12 Design 2B: (Doc + Carb) × 18	T + L[b]	2093	1080 (52)	1203 (57)	52	DFS, OS, and safety
							T→L[b]	2091	1078 (52)	1205 (58)	T12→L34	
							T	2097	1072 (51)	1200 (57)	52	
APHINITY (Von Minckwitz 2017)	III	4805	3.8 y	None	None	FEC×(9–12) + Doc/Pal × 12; or AC/EC × (8–12) + Doc/Pal × 12; or (Doc + Carb)×18	T + P	2400	1503 (63)	1536 (64)	52	IDFS, DFS, OS, and safety
							T	2405	1502 (62)	1546 (64)		
Neoadjuvant setting												
EORTC 10054 (Bonnefoi 2014)	IIb	128	NR	Doc × 9 + FEC × 9	L + T	NR	T	52	33 (64)	25 (48)	9	pCR (breast + nodes), pCR (breast), response rates, and safety
					T			53	36 (68)	27 (51)		
CALGB 40601 (Carey 2016)	III	295	NR	wP × 16	L + T	AC × (8–12)	T	117	NR	69 (59)	16	pCR (breast + nodes) and safety
					T			118		70 (59)		
NeoALTTO* (Baselga 2012; de Azambuja 2014)	III	455	3.84 y	wP × 12w	L + T	FEC × 9	T + L	152	UNK	77 (51)	52	pCR (breast + nodes), pCR (breast), ORR, safety, DFS, EFS, and OS
					T		T	149		75 (50)		
CHER-LOB* (Guarneri 2012; Guarneri 2015)	IIb	121	NR	wP × 12 + FEC × 12	L + T	NR	T	46	NR	28 (61)	26	pCR (breast + nodes) and clinical objective responses
					T			36		21 (58)		
NeoSphere* (Gianni 2012; Gianni 2016)	II	417	5 y	D × 12	T + P	FEC × 9	T	107	75 (70)	50 (47)	12	pCR(breast) and pCR (breast + nodes) Clinical response rate, safety, PFS, and DFS
				None	T + P	D × 12 <+ FEC × 9		107	75 (70)	51 (48)		
				D × 12	T	FEC×9		107	75 (70)	50 (47)		
LPT109096 (Holmes 2013)	II	100	NR	FEC × 12 + wP × 12	T + L	UNK	UNK	33	20 (61)	20 (61)	26	pCR (breast + nodes), clinical complete response (CCR), and safety
					T			33	15 (45)	15 (45)		

TABLE 1: Continued.

Study	Phase	N	MF	Neoadjuvant treatment		Adjuvant treatment							Outcomes
				Chemotherapy (wks)	Anti-HER2 therapy	Chemotherapy (wks)	Anti-HER2 therapy	Arms	LN+ patients (%)	HR+ patients (%)	Duration# (wks)		
NSABP B-41*	III	519	5 y	AC × 12 + wP × 12	T + L	None	T	174	85 (49)	108 (62)	12		pCR(breast), pCR (breast + nodes), clinical complete response, safety, and OS
(Robidoux 2013; Robidoux 2016)					T			181	92 (51)	122 (67)			

MF: median follow-up; A: anthracycline; FEC: fluorouracil + epirubicin + cyclophosphamide; Doc: docetaxel; Carb: carboplatin; Pal: paclitaxel; wP: weekly paclitaxel; wk: week; HR+: hormone receptor positive; LN+: lymph node positive; T: trastuzumab; L: lapatinib; P: pertuzumab; N: neratinib; T + L: trastuzumab plus lapatinib; T ⟶ L: trastuzumab followed by lapatinib; NR: unreported; UNK: unknown. #Duration of dual anti-HER2 therapy. *Studies with more than one publication. aIn the ExteNET trial (Martin 2017), lymph node- (LN-) positive patients were divided into two subgroups, 1–3 positive LN (Martin1 2017) and ≥4 positive LN (Martin2 2017), for the subgroup analysis of LN status. b In the ALTTO trial (Moreno-Aspitia 2017), there were two experimental groups (trastuzumab plus lapatinib group (Moreno-Aspitia 2017) and trastuzumab followed by lapatinib group (Moreno-Aspitia2 2017).

Study or subgroup	Log [hazard ratio]	SE	Weight (%)	Hazards ratio IV, random, 95% CI	Hazards ratio IV, random, 95% CI
de Azambuja 2014	−0.48	0.36	3.5	0.62 [0.31, 1.25]	
Moreno-Aspitia1 2017	−0.15	0.11	37.3	0.86 [0.69, 1.07]	
Moreno-Aspitia2 2017	−0.13	0.11	37.3	0.88 [0.71, 1.09]	
Robidoux 2016	−0.46	0.49	1.9	0.63 [0.24, 1.65]	
von Minckwitz 2017	−0.12	0.15	20.1	0.89 [0.66, 1.19]	
Total (95% CI)			100.0	0.86 [0.75, 0.98]	

Heterogeneity: tau^2 = 0.00; chi^2 = 1.31; df = 4 (P = 0.86); I^2 = 0%
Test for overall effect: Z = 2.29 (P = 0.02)

Favours [dual blockade]   Favours [control]

FIGURE 2: Overall survival of trastuzumab-containing dual anti-HER2 therapy, all studies. IV: inverse-variance method; random: random-effects model; Moreno-Aspitia1 2017: trastuzumab plus lapatinib group; Moreno-Aspitia2 2017: trastuzumab followed by lapatinib group.

Study or subgroup	Log [hazard ratio]	SE	Weight (%)	Hazards ratio IV, random, 95% CI	Hazards ratio IV, random, 95% CI
de Azambuja 2014	−0.25	0.26	2.8	0.78 [0.47, 1.30]	
Gianni 2016	−0.37	0.36	1.4	0.69 [0.34, 1.40]	
Martin 2017	−0.31	0.12	13.0	0.73 [0.58, 0.93]	
Moreno-Aspitia1 2017	−0.15	0.08	29.2	0.86 [0.74, 1.01]	
Moreno-Aspitia2 2017	−0.07	0.07	38.2	0.93 [0.81, 1.07]	
von Minckwitz 2017	−0.21	0.11	15.4	0.81 [0.65, 1.01]	
Total (95% CI)			100.0	0.86 [0.79, 0.93]	

Heterogeneity: tau^2 = 0.00; chi^2 = 3.89; df = 5 (P = 0.57); I^2 = 0%
Test for overall effect: Z = 3.60 (P = 0.0003)

Favours [dual blockade]   Favours [control]

FIGURE 3: Event-free survival/invasive disease-free survival of trastuzumab-containing dual anti-HER2 therapy-all studies. IV: inverse-variance method; random: random-effects model; Moreno-Aspitia1 2017: trastuzumab plus lapatinib group; Moreno-Aspitia2 2017: trastuzumab followed by lapatinib group.

4. Discussion

This meta-analysis of RCTs demonstrated that trastuzumab-containing dual anti-HER2 therapy was superior to standard trastuzumab alone therapy for HER2-positive EBC treatment, with a significant improvement in EFS/iDFS and OS.

Although dual anti-HER2 therapy has shown significant improvement in pCR in neoadjuvant treatment, our results demonstrated that the benefit of dual-targeting therapy in the neoadjuvant treatment did not extend to the long-term survival benefits, a significant DFS and OS benefit in favour of the adjuvant treatment versus the neoadjuvant treatment. Despite no substantial heterogeneity was found in all pooled analyses, differences between studies might be relevant. Firstly, differences in duration of dual-targeted treatment are as follows: all three studies included in the adjuvant setting have completed a 1-year dual anti-HER2 therapy [26, 28, 42], while there was only one of seven studies in the neoadjuvant setting [31]. Secondly, differences in included populations are as follows: in the adjuvant treatment, the population recruited in the APINITY trial and the ExteNET trial were relatively high-risk (with more LN+ patients, 63% and 77%, resp.), which were more likely to report positive

results in the adjuvant setting. However, no interaction between survival and treatment setting was observed (interaction test for EFS/iDFS and OS, p = 0.52 and 0.26). Thus, some caution is still required.

When taking hormone receptor status into consideration, several meta-analyses of neoadjuvant treatment demonstrated that the pCR rate was significantly improved in patients receiving dual HER2 block versus trastuzumab alone and higher in HR patients [43–46]. In adjuvant therapy, the APINITY and ALTTO trials suggested that the dual-targeted therapy could significantly enhance EFS/iDFS in HR patients [26, 28]. These results of previous studies seem to indicate that HR patients can benefit more from dual anti-HER2 therapy. Nonetheless, subgroup analysis of hormone receptor status in our meta-analysis found no difference in EFS/iDFS between the two groups. Even if the pCR was more pronounced in HR patients, no interaction was found (interaction test, p = 0.34). Therefore, hormone receptor status may not be a determinant of a dual-targeted selective therapy. Or as described in the CALGB 40601 study, we should pay more attention to the subtype than hormone receptor status when predicting pCR [32]. Of note, the ExteNET trial suggested that neratinib administered after

Study or subgroup	Log [hazard ratio]	SE	Weight (%)	Hazards ratio IV, random, 95% CI	Hazards ratio IV, random, 95% CI
1.6.1 T+L					
de Azambuja 2014	−0.25	0.26	2.8	0.78 [0.47, 1.30]	
Moreno-Aspitia1 2017	−0.15	0.08	29.2	0.86 [0.74, 1.01]	
Moreno-Aspitia2 2017	−0.07	0.07	38.2	0.93 [0.81, 1.07]	
Subtotal (95% CI)			70.1	0.90 [0.81, 0.99]	
Heterogeneity: tau^2 = 0.00; chi^2 = 0.87; df = 2 (P = 0.65); I^2 = 0%					
Test for overall effect: Z = 2.14 (P = 0.03)					
1.6.2 T+P					
Gianni 2016	−0.37	0.36	1.4	0.69 [0.34, 1.40]	
von Minckwitz 2017	−0.21	0.11	15.4	0.81 [0.65, 1.01]	
Subtotal (95% CI)			16.9	0.80 [0.65, 0.98]	
Heterogeneity: tau^2 = 0.00; chi^2 = 0.18; df = 1 (P = 0.67); I^2 = 0%					
Test for overall effect: Z = 2.13 (P = 0.03)					
1.6.3 T+N					
Martin 2017	−0.31	0.12	13.0	0.73 [0.58, 0.93]	
Subtotal (95% CI)			13.0	0.73 [0.58, 0.93]	
Heterogeneity: not applicable					
Test for overall effect: Z = 2.58 (P = 0.010)					
Total (95% CI)			100.0	0.86 [0.79, 0.93]	
Heterogeneity: tau^2 = 0.00; chi^2 = 3.89; df = 5 (P = 0.57); I^2 = 0%					
Test for overall effect: Z = 3.60 (P = 0.0003)					
Test for subgroup differences: chi^2 = 2.84; df = 2 (P = 0.24); I^2 = 29.6%					

FIGURE 4: Event-free survival/invasive disease-free survival stratified by type of dual HER2 blockade regimen. T: trastuzumab; L: lapatinib; P: pertuzumab; N: neratinib; IV: inverse-variance method; random: random-effects model; Moreno-Aspitia1 2017: trastuzumab plus lapatinib group; Moreno-Aspitia2 2017: trastuzumab followed by lapatinib group.

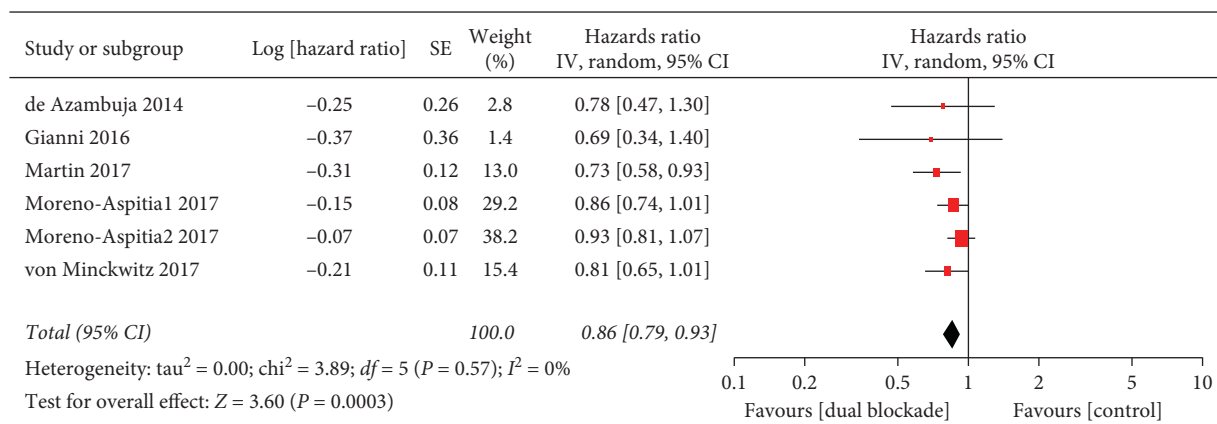trastuzumab significantly improved iDFS in hormone receptor-positive (HR+) patients with HER2-positive BC. This may be a consequence of there being no cross-resistance for neratinib and trastuzumab in the HR + patients, or the interaction of neratinib with hormones reversed the upregulation of estrogen receptors caused by trastuzumab to modify HER2 resistance [42]. With results diametrically opposite to other studies, we conducted an extra subgroup analysis excluding the ExteNET study and the results did not change.

The LN status, another important factor affecting the clinical treatment decisions, has been shown in clinical studies that LN + patients are more likely to benefit from dual-targeting therapy [24, 42], but our results suggested that, despite the more pronounced EFS/iDFS benefit in LN + patients, there was no significant interaction between survival and LN status (p = 0.18). Similarly, a recent meta-analysis assessing the optimal duration of trastuzumab treatment also showed no significant interaction between survival and HR status or LN status (p for interaction test, 0.26 and 0.60) [47]. The guidelines recommend using an interaction test for subgroup analyses, as evidenced that inappropriate subgroup-specific analysis was of low reliability and the problem may be underestimated [48]. Thus, the subgroup results should be interpreted carefully.

In addition to the above, different combination regimens of dual HER2 block might affect efficacy. Subgroup analysis of the type of dual anti-HER2 regimen revealed that OS was significantly improved with trastuzumab plus lapatinib, while the effect on EFS/iDFS did not differ significantly among the three groups. Although the OS benefit with trastuzumab plus lapatinib might be somewhat unexpected considering the negative results of the ALTTO trial, the following points in the ALTTO trial should be noted, except for the unreported final OS results of the ExteNET trial: First, the recruited patients were designed for DFS, with a low risk of recurrence (more LN− (40%) and HR+ (57%) patients than the other included trials), which may explain the lower-than-expected DFS event [40]. Second, a time-driven analysis was conducted to obtain early results rather than a more mature event-driven analysis [49]. Third, due to the toxicity of lapatinib, the lapatinib group was closed early and the proportion of patients who completed the planned dose in the dual-targeting group was lower. Finally, studies demonstrated that intermittent administration of lapatinib is more effective than continuous administration [50, 51]. All of above may affect statistical power and result in negative results [49, 52]. Notably, in the ALTTO trial, a protocol modification required $p \leq 0.25$ because of the early closure of the lapatinib group, while we considered $p \leq 0.05$ to be

Study or subgroup	Log [hazard ratio]	SE	Weight (%)	Hazards ratio IV, random, 95% CI	Hazards ratio IV, random, 95% CI
1.8.1 LN+					
Martin1 2017	−0.29	0.18	18.9	0.75 [0.53, 1.06]	
Martin2 2017	−0.4	0.19	16.9	0.67 [0.46, 0.97]	
von Minckwitz 2017	−0.26	0.11	50.5	0.77 [0.62, 0.96]	
Subtotal (95% CI)			86.3	0.75 [0.63, 0.88]	

Heterogeneity: tau^2 = 0.00; chi^2 = 0.41; df = 2 (P = 0.82); I^2 = 0%

Test for overall effect: Z = 3.49 (P = 0.0005)

1.8.2 LN−					
Martin 2017	−0.19	0.36	4.7	0.83 [0.41, 1.67]	
von Minckwitz 2017	0.12	0.26	9.0	1.13 [0.68, 1.88]	
Subtotal (95% CI)			13.7	1.01 [0.67, 1.53]	

Heterogeneity: tau^2 = 0.00; chi^2 = 0.49; df = 1 (P = 0.49); I^2 = 0%

Test for overall effect: Z = 0.07 (P = 0.95)

Total (95% CI)			100.0	0.78 [0.67, 0.91]	

Heterogeneity: tau^2 = 0.00; chi^2 = 2.73; df = 4 (P = 0.60); I^2 = 0%

Test for overall effect: Z = 3.22 (P = 0.001)

Test for subgroup differences: chi^2 = 1.84; df = 1 (P = 0.18); I^2 = 45.6%

0.2   0.5   1   2   5
Favours [dual blockade]   Favours [control]

(a)

Study or subgroup	Log [hazard ratio]	SE	Weight (%)	Hazards ratio IV, random, 95% CI	Hazards ratio IV, random, 95% CI
1.7.1 HR+					
de Azambuja 2014	−0.04	0.38	1.3	0.96 [0.46, 2.02]	
Martin 2017	−0.51	0.17	6.5	0.60 [0.43, 0.84]	
Moreno-Aspitia1 2017	−0.09	0.1	18.7	0.91 [0.75, 1.11]	
Moreno-Aspitia2 2017	−0.11	0.1	18.7	0.90 [0.74, 1.09]	
von Minckwitz 2017	−0.15	0.14	9.5	0.86 [0.65, 1.13]	
Subtotal (95% CI)			54.6	0.85 [0.74, 0.97]	

Heterogeneity: tau^2 = 0.01; chi^2 = 5.08; df = 4 (P = 0.28); I^2 = 21%

Test for overall effect: Z = 2.39 (P = 0.02)

1.7.2 HR−					
de Azambuja 2014	−0.43	0.35	1.5	0.65 [0.33, 1.29]	
Martin 2017	−0.05	0.18	5.8	0.95 [0.67, 1.35]	
Moreno-Aspitia1 2017	−0.22	0.11	15.4	0.80 [0.65, 1.00]	
Moreno-Aspitia2 2017	−0.03	0.11	15.4	0.97 [0.78, 1.20]	
von Minckwitz 2017	−0.27	0.16	7.3	0.76 [0.56, 1.04]	
Subtotal (95% CI)			45.4	0.86 [0.76, 0.98]	

Heterogeneity: tau^2 = 0.00; chi^2 = 3.11; df = 4 (P = 0.54); I^2 = 0%

Test for overall effect: Z = 2.32 (P = 0.02)

Total (95% CI)			100.0	0.86 [0.79, 0.93]	

Heterogeneity: tau^2 = 0.00; chi^2 = 8.19; df = 9 (P = 0.52); I^2 = 0%

Test for overall effect: Z = 3.54 (P = 0.0004)

Test for subgroup differences: chi^2 = 0.03; df = 1 (P = 0.86); I^2 = 0%

0.01   0.1   1   10   100
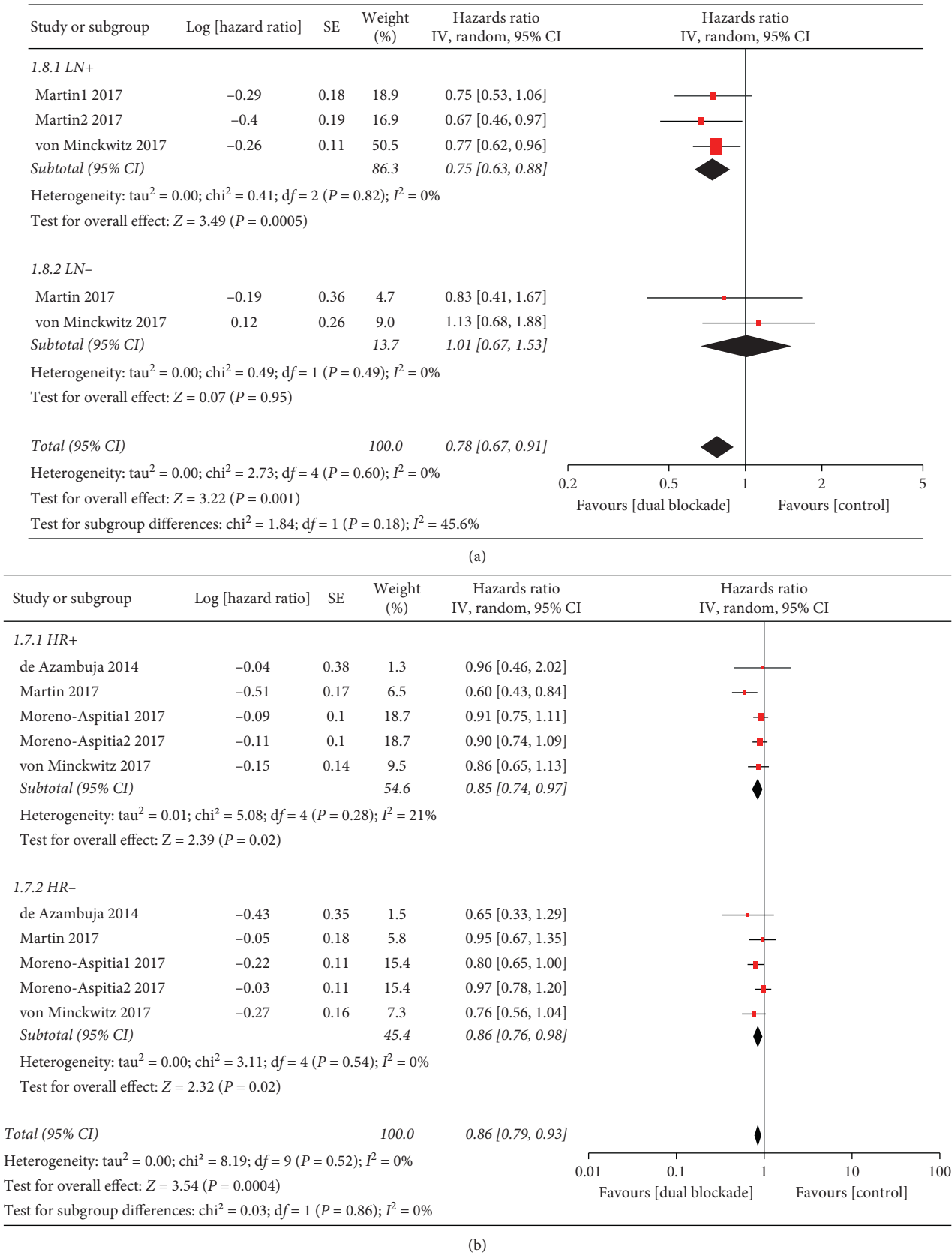Favours [dual blockade]   Favours [control]

(b)

FIGURE 5: Subgroup analyses of event-free survival/invasive disease-free survival (EFS/iDFS). (a) EFS/iDFS stratified by lymph node status. LN+: lymph node positive; LN−: lymph node negative; Martin1 2017: subgroup of 1–3 positive LN; Martin2 2017: subgroup of ≥4 positive LN. (b) EFS/iDFS stratified by hormone receptor status. HR+: hormone receptor positive; HR−: hormone receptor negative; IV: inverse-variance method; random: random-effects model; Moreno-Aspitia1 2017: trastuzumab plus lapatinib group; Moreno-Aspitia2 2017: trastuzumab followed by lapatinib group.

Study or subgroup	Dual blockade Events	Total	Control Events	Total	Weight (%)	Risk ratio IV, random, 95% CI	Risk ratio IV, random, 95% CI
Baselga 2012	1	152	1	149	4.1	0.98 [0.06, 15.53]	
Bonnefoi 2014	3	50	0	53	3.7	7.41 [0.39, 139.97]	
Carey 2016	4	117	4	118	13.0	1.01 [0.26, 3.94]	
Gianni 2012	3	107	1	107	5.9	3.00 [0.32, 28.39]	
Guarneri 2012	0	46	1	36	3.2	0.26 [0.01, 6.26]	
Piccart-Gebhart 2016	972	4137	518	2076	43.0	0.94 [0.86, 1.03]	
Robidoux 2013	1	173	7	178	6.7	0.15 [0.02, 1.18]	
von Minckwitz 2017	15	2364	6	2405	20.4	2.54 [0.99, 6.54]	
Total (95% CI)		7146		5122	100.0	1.14 [0.63, 2.05]	
Total events	999		538				

Heterogeneity: tau^2 = 0.21; chi^2 = 10.81; df = 7 (P = 0.15); I^2 = 35%

Test for overall effect: Z = 0.44 (P = 0.66)

0.01   0.1   1   10   100

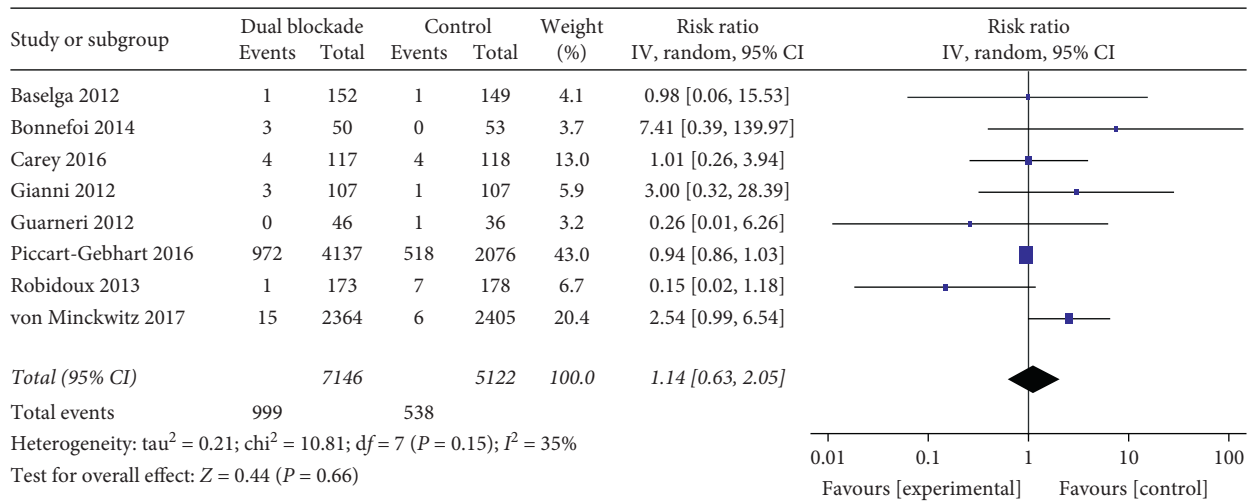Favours [experimental]   Favours [control]

FIGURE 6: Cardiac toxicity of trastuzumab-containing dual anti-HER2 therapy-all studies. IV: inverse-variance method; random: random-effects model.

statistically significant [28]. And it is statistically possible that the pooled analysis showed a marginally significant result after expanding the sample size by integrating several trials that are close to meaningful. Additionally, the meta-analysis by Debiasi et al. [53] also found that chemotherapy plus trastuzumab plus lapatinib was probably the first choice for improving OS compared to chemotherapy plus trastuzumab with a posterior probability of 62.47%. Trastuzumab plus neratinib was the best strategy for DFS, with a posterior probability of 50.55%. These results coincided with ours, but our meta-analysis also included the mature OS results of the APINITY trial. It seems that there might be differences among the three dual anti-HER2 regimens in terms of EFS/iDFS and OS, but no significant interactions were observed (p = 0.24 and 0.80, resp.). More RCTs are needed to confirm the best combination regimen due to the limited number of trials included in each subgroup.

Regarding the toxicities, the risk of cardiac toxicity did not increase, as described in other meta-analyses [43, 45, 46], which increases our confidence in using dual-targeted therapy. However, the incidence of grade 3/4 diarrhea, hepatic toxicity, nausea and vomiting, and skin disorders was significantly increased. Subgroup analysis of dual anti-HER2 regimen showed that the toxicities in the lapatinib group were mainly diarrhea, hepatic toxicity, and skin disorders, and the main toxicities for the neratinib group were diarrhea, nausea and vomiting, and fatigue, while for the pertuzumab group the main toxicity was diarrhea. Almost all trials that contained treatment with lapatinib reported a dose reduction, termination of treatment, and even early closure of the treatment group due to the high risk of adverse events (AEs) that can also be seen in other published meta-analyses [45, 54, 55]. Conversely, most of the cases of diarrhea reported in the neratinib-containing group were of low grade and were preventable and tolerable despite the high incidence. The risk of AEs in the pertuzumab-containing group was significantly lower than that in the lapatinib group and the neratinib group. Therefore, we

believe that trastuzumab plus lapatinib would be the most effective regimen if the patients could tolerant the toxicity. If they cannot, then trastuzumab plus pertuzumab or plus neratinib would be the preferred options for HER2-positive EBC after weighing the effects and safety. We are still waiting for the final OS result of the ExteNET trial and more trials using dual HER2 blocking with trastuzumab plus pertuzumab or plus neratinib.

Our manuscripts collected comprehensive and latest clinical data to make up for the deficiencies of previous studies and present the most cutting-edge results in this field. We compared dual anti-HER2 therapy with the current standard care (trastuzumab alone) for treating HER2-positive EBC and comprehensively evaluated efficacy and safety, the neoadjuvant and adjuvant setting, and corresponding subgroup analyses to look for the populations that would most benefit to identify crucial personalize therapy.

Nevertheless, shortcomings remain. Firstly, the heterogeneous nature of the patients, the clinical settings, and the drugs in this meta-analysis may reduce reliability. However, we conducted the pooled analyses, several correlation subgroup analyses, and sensitivity analyses, and the results did not show any significant heterogeneity. Secondly, for data available for the regimen with trastuzumab plus neratinib or plus pertuzumab, hormone receptor status and LN status were limited. And neratinib was administered after completion of trastuzumab-based adjuvant therapy rather than being used simultaneously in the ExteNET trial; further RCTs are still needed to focus on trastuzumab plus pertuzumab or plus neratinib regimens and LN status and hormone receptor status to improve our understanding. Finally, EFS/iDFS and OS can be affected by subsequent adjuvant therapy such as the regimens and duration of treatment.

5. Conclusions

We conclude that the trastuzumab-containing dual HER2 block is superior to standard trastuzumab alone for patients

with HER2-positive EBC. Although the dual HER2 block was associated with a higher risk of grade 3/4 AEs, especially in the lapatinib group, there was no increase in cardiotoxicity. Trastuzumab combined with lapatinib achieved the greatest OS benefit but is accompanied by higher AEs. Weighing the pros and cons, trastuzumab plus pertuzumab or plus neratinib is the preferred choice with substantial benefit and lower toxicity, a result still waiting for the final OS results of the ExteNET trial. Notably, the survival was independent of hormone receptor status, and the correlation between survival and LN status should be interpreted cautiously. Further investigations are needed to determine the best dual anti-HER2 regimen and the subgroup populations that will benefit most.

Data Availability

The data used to support the findings of this study are included within the supplementary information files.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Liuwen Yu, Fangmeng Fu, and Jing Li were involved in the conception and design of the study. Liuwen Yu, Fangmeng Fu, Jing Li, Yuxiang Lin, Qian Mei, and Jinxing Lv participated in the study search, selection, and evaluation. Liuwen Yu, Bangwei Zeng, Meng Huang, and Jing Li performed data extraction and analysis. Liuwen Yu drafted the manuscript, and all authors participated in the writing of the manuscript and interpretation of the results. All authors read and approved the final version.

Supplementary Materials

Additional file 1: Appendix 1: definitions of outcomes. Appendix 2: characteristics of included studies. Appendix 3: risk of bias assessment. Additional file 2: Figure S1: forest plots for all outcomes. Figure S2: sensitivity analyses. Figure S3: publication bias. (*Supplementary Materials*)

References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA: A Cancer Journal for Clinicians*, vol. 68, no. 6, pp. 394–424, 2018.

[2] C. M. Perou, T. Sørlie, M. B. Eisen et al., "Molecular portraits of human breast tumours," *Nature*, vol. 406, no. 6797, pp. 747–752, 2000.

[3] D. Slamon, G. Clark, S. Wong, W. Levin, A. Ullrich, and W. McGuire, "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene," *Science*, vol. 235, no. 4785, pp. 177–182, 1987.

[4] D. Slamon, W. Godolphin, L. Jones et al., "Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer," *Science*, vol. 244, no. 4905, pp. 707–712, 1989.

[5] T. Sorlie, C. M. Perou, R. Tibshirani et al., "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications," *Proceedings of the National Academy of Sciences*, vol. 98, no. 19, pp. 10869–10874, 2001.

[6] M. A. Olayioye, R. M. Neve, H. A. Lane, and N. E. Hynes, "New EMBO members' review: the ErbB signaling network: receptor heterodimerization in development and cancer," *The EMBO Journal*, vol. 19, no. 13, pp. 3159–3167, 2000.

[7] S. Balduzzi, S. Mantarro, V. Guarneri et al., "Trastuzumab-containing regimens for metastatic breast cancer," *Cochrane Database of Systematic Reviews*, vol. 6, 2014.

[8] L. Moja, L. Tagliabue, S. Balduzzi et al., "Trastuzumab containing regimens for early breast cancer," *Cochrane Database of Systematic Reviews*, vol. 4, 2012.

[9] P. Conte, A. Frassoldati, G. Bisagni et al., "9 weeks vs 1 year adjuvant trastuzumab in combination with chemotherapy: final results of the phase III randomized Short-HER study," *Journal of Clinical Oncology*, vol. 35, 2018.

[10] D. J. Slamon, W. Eiermann, N. J. Robert et al., "Abstract S5-04: Ten year follow-up of BCIRG-006 comparing doxorubicin plus cyclophosphamide followed by docetaxel (AC⟶T) with doxorubicin plus cyclophosphamide followed by docetaxel and trastuzumab (AC⟶TH) with docetaxel, carboplatin and trastuzumab (TCH) in HER2+ early breast cancer," *Cancer Research*, vol. 76, no. 4, Article ID S5-04-S5-04, 2016.

[11] D. Cameron, M. J. Piccart-Gebhart, R. D. Gelber et al., "11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin adjuvant (HERA) trial," *The Lancet*, vol. 389, no. 10075, pp. 1195–1205, 2017.

[12] E. A. Perez, E. H. Romond, V. J. Suman et al., "Trastuzumab plus adjuvant chemotherapy for human epidermal growth factor receptor 2-positive breast cancer: planned joint analysis of overall survival from NSABP B-31 and NCCTG N9831," *Journal of Clinical Oncology*, vol. 32, no. 33, pp. 3744–3752, 2014.

[13] Q. Ryan, A. Ibrahim, M. H. Cohen et al., "FDA drug approval summary: lapatinib in combination with capecitabine for previously treated metastatic breast cancer that overexpresses HER-2," *The Oncologist*, vol. 13, no. 10, pp. 1114–1119, 2008.

[14] L. Amiri-Kordestani, S. Wedam, L. Zhang et al., "First FDA approval of neoadjuvant therapy for breast cancer: pertuzumab for the treatment of patients with HER2-positive breast cancer," *Clinical Cancer Research*, vol. 20, no. 21, pp. 5359–5364, 2014.

[15] H. Singh, A. J. Walker, L. Amiri-Kordestani et al., "U.S. food and drug administration approval: neratinib for the extended adjuvant treatment of early-stage HER2-positive breast cancer," *Clinical Cancer Research*, vol. 24, no. 15, pp. 3486–3491, 2018.

[16] C. W. Adams, D. E. Allison, K. Flagella et al., "Humanization of a recombinant monoclonal antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab," *Cancer Immunology, Immunotherapy*, vol. 55, no. 6, pp. 717–727, 2006.

[17] N. U. Lin and E. P. Winer, "New targets for therapy in breast cancer: small molecule tyrosine kinase inhibitors," *Breast Cancer Research*, vol. 6, no. 5, p. 204, 2004.

[18] J. W. Park, M. C. Liu, D. Yee et al., "Adaptive randomization of neratinib in early breast cancer," *New England Journal of Medicine*, vol. 375, no. 1, pp. 11–22, 2016.

[19] J. Veeraraghavan, C. De Angelis, R. Mao et al., "A combinatorial biomarker predicts pathologic complete response to neoadjuvant lapatinib and trastuzumab without chemotherapy in patients with HER2+ breast cancer," *Annals of Oncology*, vol. 30, no. 6, pp. 927–933, 2019.

[20] H. K. Kim, K. H. Park, Y. Kim et al., "Discordance of the PAM50 intrinsic subtypes compared with immunohisto-chemistry-based surrogate in breast cancer patients: potential implication of genomic alterations of discordance," *Cancer Research and Treatment*, vol. 51, no. 2, pp. 737–747, 2019.

[21] C. Denkert, S. Loibl, A. Noske et al., "Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer," *Journal of Clinical Oncology*, vol. 28, no. 1, pp. 105–113, 2010.

[22] R. Salgado, C. Denkert, C. Campbell et al., "Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in HER2-positive early-stage breast cancer treated with lapatinib and trastuzumab," *JAMA Oncology*, vol. 1, no. 4, pp. 448–454, 2015.

[23] M. Ignatiadis, G. Van den Eynden, S. Roberto et al., "Tumor-infiltrating lymphocytes in patients receiving trastuzumab/pertuzumab-based chemotherapy: a TRYPHAENA sub-study," *JNCI: Journal of the National Cancer Institute*, vol. 111, no. 1, pp. 69–77, 2018.

[24] L. Gianni, T. Pienkowski, Y.-H. Im et al., "5-year analysis of neoadjuvant pertuzumab and trastuzumab in patients with locally advanced, inflammatory, or early-stage HER2-positive breast cancer (NeoSphere): a multicentre, open-label, phase 2 randomised trial," *The Lancet Oncology*, vol. 17, no. 6, pp. 791–800, 2016.

[25] J. Baselga, I. Bradbury, H. Eidtmann et al., "Lapatinib with trastuzumab for HER2-positive early breast cancer (Neo-ALTTO): a randomised, open-label, multicentre, phase 3 trial," *The Lancet*, vol. 379, no. 9816, pp. 633–640, 2012.

[26] G. von Minckwitz, M. Procter, E. de Azambuja et al., "Adjuvant pertuzumab and trastuzumab in early HER2-positive breast cancer," *New England Journal of Medicine*, vol. 377, no. 2, pp. 122–131, 2017.

[27] National Comprehensive Cancer Network, *Breast Cancer-NCCN Clinical Practice Guidelines in Oncology*, National Comprehensive Cancer Network, Plymouth Meeting, PA, USA, 2018.

[28] M. Piccart-Gebhart, E. Holmes, J. Baselga et al., "Adjuvant lapatinib and trastuzumab for early human epidermal growth factor receptor 2-positive breast cancer: results from the randomized phase III adjuvant lapatinib and/or trastuzumab treatment optimization trial," *Journal of Clinical Oncology*, vol. 34, no. 10, pp. 1034–1042, 2016.

[29] J. P. Higgins, *Cochrane Handbook for Systematic Reviews of Interventions*, John Wiley & Sons, Hoboken, NJ, USA, 2010.

[30] C. B. Begg and M. Mazumdar, "Operating characteristics of a rank correlation test for publication bias," *Biometrics*, vol. 50, no. 4, pp. 1088–1101, 1994.

[31] E. de Azambuja, A. P. Holmes, M. Piccart-Gebhart et al., "Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response," *The Lancet Oncology*, vol. 15, no. 10, pp. 1137–1146, 2014.

[32] L. A. Carey, D. A. Berry, C. T. Cirrincione et al., "Molecular heterogeneity and response to neoadjuvant human epidermal growth factor receptor 2 targeting in CALGB 40601, a randomized phase III trial of paclitaxel plus trastuzumab with or without lapatinib," *Journal of Clinical Oncology*, vol. 34, no. 6, pp. 542–549, 2016.

[33] V. Guarneri, A. Frassoldati, A. Bottini et al., "Preoperative chemotherapy plus trastuzumab, lapatinib, or both in human epidermal growth factor receptor 2-positive operable breast cancer: results of the randomized phase II CHER-LOB study,"

[34] V. Guarneri, M. V. Dieci, A. Frassoldati et al., "Prospective biomarker analysis of the randomized CHER-LOB study evaluating the dual anti-HER2 treatment with trastuzumab and lapatinib plus chemotherapy as neoadjuvant therapy for HER2-positive breast cancer," *The Oncologist*, vol. 20, no. 9, pp. 1001–1010, 2015.

[35] F. A. Holmes, V. Espina, L. A. Liotta et al., "Pathologic complete response after preoperative anti-HER2 therapy correlates with alterations in PTEN, FOXO, phosphorylated Stat5, and autophagy protein signaling," *BMC Research Notes*, vol. 6, no. 1, p. 507, 2013.

[36] A. Robidoux, G. Tang, P. Rastogi et al., "Lapatinib as a component of neoadjuvant therapy for HER2-positive operable breast cancer (NSABP protocol B-41): an open-label, randomised phase 3 trial," *The Lancet Oncology*, vol. 14, no. 12, pp. 1183–1192, 2013.

[37] A. Robidoux, G. Tang, P. Rastogi et al., "Evaluation of lapatinib as a component of neoadjuvant therapy for HER2+ operable breast cancer: 5-year outcomes of NSABP protocol B-41," *Journal of Clinical Oncology*, vol. 34, 2017.

[38] H. Bonnefoi, W. Jacot, M. Saghatchian et al., "Neoadjuvant treatment with docetaxel plus lapatinib, trastuzumab, or both followed by an anthracycline-based chemotherapy in HER2-positive breast cancer: results of the randomised phase II EORTC 10054 study," *Annals of Oncology*, vol. 26, no. 2, pp. 325–332, 2015.

[39] L. Gianni, T. Pienkowski, Y.-H. Im et al., "Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial," *The Lancet Oncology*, vol. 13, no. 1, pp. 25–32, 2012.

[40] A. Moreno-Aspitia, E. M. C. Holmes, C. Jackisch et al., "Updated results from the phase III ALTTO trial (BIG 2-06; NCCTG (Alliance) N063D) comparing one year of anti-HER2 therapy with lapatinib alone (L), trastuzumab alone (T), their sequence (T⟶ L) or their combination (L+ T) in the adjuvant treatment of HER2-positive early breast cancer," *Journal of Clinical Oncology*, 2017.

[41] A. Chan, S. Delaloge, F. A. Holmes et al., "Neratinib after trastuzumab-based adjuvant therapy in patients with HER2-positive breast cancer (ExteNET): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial," *The Lancet Oncology*, vol. 17, no. 3, pp. 367–377, 2016.

[42] M. Martin, F. A. Holmes, B. Ejlertsen et al., "Neratinib after trastuzumab-based adjuvant therapy in HER2-positive breast cancer (ExteNET): 5-year analysis of a randomised, double-blind, placebo-controlled, phase 3 trial," *The Lancet Oncology*, vol. 18, no. 12, pp. 1688–1700, 2017.

[43] A. Nagayama, T. Hayashida, H. Jinno et al., "Comparative effectiveness of neoadjuvant therapy for HER2-positive breast cancer: a network meta-analysis," *JNCI: Journal of the National Cancer Institute*, vol. 106, no. 9, 2014.

[44] M. Clavarezza, M. Puntoni, A. Gennari et al., "Dual block with lapatinib and trastuzumab versus single-agent trastuzumab combined with chemotherapy as neoadjuvant treatment of HER2-positive breast cancer: a meta-analysis of randomized trials," *Clinical Cancer Research*, vol. 22, no. 18, pp. 4594–4603, 2016.

[45] A. Nakashoji, T. Hayashida, T. Yokoe et al., "The updated network meta-analysis of neoadjuvant therapy for HER2-positive breast cancer," *Cancer Treatment Reviews*, vol. 62, pp. 9–17, 2018.

Journal of Clinical Oncology, vol. 30, no. 16, pp. 1989–1995, 2012.

[46] E. Bria, L. Carbognin, J. Furlanetto et al., "Impact of neo-adjuvant single or dual HER2 inhibition and chemotherapy backbone upon pathological complete response in operable and locally advanced breast cancer: sensitivity analysis of randomized trials," *Cancer Treatment Reviews*, vol. 40, no. 7, pp. 847–856, 2014.

[47] L. Chen, W. Zhou, X. Hu, M. Yi, C. Ye, and G. Yao, "Short-duration versus 1-year adjuvant trastuzumab in early HER2 positive breast cancer: a meta-analysis of randomized controlled trials," *Cancer Treatment Reviews*, vol. 75, pp. 12–19, 2019.

[48] S. T. Brookes, E. Whitely, M. Egger, G. D. Smith, P. A. Mulheran, and T. J. Peters, "Subgroup analyses in randomized trials: risks of subgroup-specific analyses;: power and sample size for the interaction test," *Journal of Clinical Epidemiology*, vol. 57, no. 3, pp. 229–236, 2004.

[49] L. De Mattos-Arruda, R. Shen, J. S. Reis-Filho, and J. Cortés, "Translating neoadjuvant therapy into survival benefits: one size does not fit all," *Nature Reviews Clinical Oncology*, vol. 13, no. 9, pp. 566–579, 2016.

[50] D. N. Amin, N. Sergina, D. Ahuja et al., "Resiliency and vulnerability in the HER2-HER3 tumorigenic driver," *Science Translational Medicine*, vol. 2, no. 16, p. 16ra7, 2010.

[51] A. J. Chien, P. N. Munster, M. E. Melisko et al., "Phase I dose-escalation study of 5-day intermittent oral lapatinib therapy in patients with human epidermal growth factor receptor 2-overexpressing breast cancer," *Journal of Clinical Oncology*, vol. 32, no. 14, pp. 1472–1479, 2014.

[52] G. Bianchini, "Is trastuzumab as a single agent obsolete in early breast cancer? No," *The Breast*, vol. 43, pp. 142–145, 2019.

[53] M. Debiasi, C. A. Polanczyk, P. Ziegelmann et al., "Efficacy of anti-HER2 agents in combination with adjuvant or neo-adjuvant chemotherapy for early and locally advanced HER2-positive breast cancer patients: a network meta-analysis," *Frontiers in Oncology*, vol. 8, p. 156, 2018.

[54] A. Valachis, A. Nearchou, P. Lind, and D. Mauri, "Lapatinib, trastuzumab or the combination added to preoperative chemotherapy for breast cancer: a meta-analysis of randomized evidence," *Breast Cancer Research and Treatment*, vol. 135, no. 3, pp. 655–662, 2012.

[55] Y. Xin, W. W. Guo, Q. Huang et al., "Effects of lapatinib or trastuzumab, alone and in combination, in human epidermal growth factor receptor 2-positive breast cancer: a meta-analysis of randomized controlled trials," *Cancer Medicine*, vol. 5, no. 12, pp. 3454–3463, 2016.

Review Article

Somatic Mutations in *HER2* and Implications for Current Treatment Paradigms in *HER2*-Positive Breast Cancer

Maria Gaibar [ID]**, Laura Beltrán** [ID]**, Alicia Romero-Lorca** [ID]**, Ana Fernández-Santander** [ID]**, and Apolonia Novillo** [ID]

Faculty of Biomedical Sciences and Health, Universidad Europea de Madrid, C/Tajo, S/N, 28670 Villaviciosa de Odón, Madrid, Spain

Correspondence should be addressed to Apolonia Novillo; apolonia.novillo@universidadeuropea.es

In one of every four or five cases of breast cancer, the human epidermal growth factor receptor-2 (*HER2*) gene is overexpressed. These carcinomas are known as HER2-positive. HER2 overexpression is linked to an aggressive phenotype and a lower rate of disease-free and overall survival. Drugs such as trastuzumab, pertuzumab, lapatinib, neratinib, and the more recent afatinib target the deregulation of HER2 expression. Some authors have attributed somatic mutations in HER2, a role in resistance to anti-HER2 therapy as differential regulation of HER2 has been observed among patients. Recently, studies in metastatic ER + tumors suggest that some HER2 mutations emerge as a mechanism of acquired resistance to endocrine therapy. In an effort to identify possible biomarkers of the efficacy of anti-HER2 therapy, we here review the known single-nucleotide polymorphisms (SNPs) of the HER2 gene found in HER2-positive breast cancer patients and their relationship with clinical outcomes. Information was recompiled on 11 somatic HER2 SNPs. Seven polymorphisms are located in the tyrosine kinase domain region of the gene contrasting with the low number of mutations found in extracellular and transmembrane areas. HER2-positive patients carrying S310F, S310Y, R678Q, D769H, or I767M mutations seem good candidates for anti-HER2 therapy as they show favorable outcomes and a good response to current pharmacological treatments. Carrying the L755S or D769Y mutation could also confer benefits when receiving neratinib or afatinib. By contrast, patients with mutations L755S, V842I, K753I, or D769Y do not seem to benefit from trastuzumab. Resistance to lapatinib has been reported in patients with L755S, V842I, and K753I. These data suggest that exploring HER2 SNPs in each patient could help individualize anti-HER2 therapies. Advances in our understanding of the genetics of the HER2 gene and its relations with the efficacy of anti-HER2 treatments are needed to improve the outcomes of patients with this aggressive breast cancer.

1. Introduction

Breast cancer is the most common cancer type worldwide and is considered a heterogeneous genomic disease in terms of molecular markers, prognosis, and treatments [1, 2]. At the molecular level, at least five clinical subtypes have been defined: hormone receptor-positive (luminal A and luminal B), human epidermal growth factor receptor-2 (HER2-positive), basal-like, normal-like, and triple-negative breast cancer (TNBC) [2–4]. Based on this classification, the oncologist is able to prescribe the best endocrine therapy, chemotherapy (alone or combined), and/or HER2-targeted therapy. About 20–25% of all breast cancers overexpress human epidermal growth factor receptor-2 (HER2) and are referred to as HER2-positive. HER2 overexpression is linked to an aggressive phenotype resulting in reduced disease-free and overall survival compared with other breast cancer subtypes, and different strategies have been developed to try to block this receptor [5–9]. According to clinical data, HER2-targeted therapy significantly improves the survival of breast cancer patients showing HER2 overexpression. However, recent data suggest the presence of oncogenic mutations in *HER2* affects clinical outcome in HER2-positive breast cancer patients [10].

In 1983, the receptor tyrosine kinase 2 gene (*ERBB2* or newly named *HER2*) was cloned [11]. This gene is located on the short arm of chromosome 17 and its product is the glycoprotein, HER2, which has several functional domains (Figure 1) that resemble those of other members of the tyrosine kinase family (HER1, HER3, and HER4): an extracellular domain (ECD, containing four subdomains), a transmembrane domain (TMD), an intracellular region that consists of a juxtamembrane domain (JMD), and a tyrosine kinase domain (TKD) [12]. HER2 is an atypical member of the ERBB family because it has no known ligand and its ECD constitutively adopts an open conformation [13]. This has led several authors to suggest a role of HER2 as coreceptor [14]. HER2 preferentially heterodimerizes with ligand bound untethered (open) HER3 or with HER4 and HER1, thereby affecting the downstream signaling of these receptors. In overexpressing cells, HER2 forms homodimers that are capable of signaling [13, 15, 16]. HER2 promotes oncogenic signaling by modulating the expression and activity of proteins controlling cell proliferation, differentiation, death, migration, and angiogenesis, activating specific PI3K/Akt (phosphatidylinositol 3-kinase/Akt, also known as PKB, protein kinase B) and MAPK (mitogen-activated protein kinase) pathways (Figure 2). Unlike other ERBB receptors, HER2 remains on the cell surface for prolonged periods after being activated to signal, which contributes to its ability to transform cells when overexpressed. New findings in breast cancer cells indicate that plasma membrane calcium ATPase2 (PMCA2) is vital for the localization of HER2 and its partners, EGFR and HER3, to activate membrane signaling domains contributing to HER2's ability to transform cells when overexpressed and prevent HER2 internalization after receptor stimulation and it sustains downstream signal transduction. This means that targeting PMCA2-HER2 interactions could be a new therapeutic approach [17]. Recently, HER2 and the cannabinoid receptor CB_2R have been described to physically interact. In effect, the expression of heteromers (HER2-CB_2R) has been correlated with a poor prognosis, while their disruption promotes an antitumor response suggesting these heteromers could be used as therapeutic targets and prognostic tools in HER2-positive breast cancer [18].

HER2 gene amplification, or protein overexpression, is still considered a major mechanism of HER2-driven tumorigenesis and is used as a main predictive biomarker to identify patients who might benefit from therapy with anti-HER2 agents. There are, thus, many different cancer drugs approved by the US Food and Drug Administration (FDA) that target the deregulation of HER2, including monoclonal antibodies, antibody-drug conjugates, and small-molecule TKIs (tyrosine kinase inhibitors), such as trastuzumab, pertuzumab, lapatinib, trastuzumab-emtansine (T-DM1), and neratinib [19–21], as well as others under investigation such as afatinib [7, 22–25] (Table 1). Molecular studies have shown that HER2-positive breast cancers are heterogeneous and that the different tumors may be classified as HER2-enriched or luminal molecular subtypes based on estrogen receptor expression (ER), with implications in their response to targeted therapies [26]. Furthermore, *HER2* mutations are

identified in 4% of breast cancer patients; these mutations are independently associated with HER2 amplification status, occurring in both hormone receptor (HR)-positive/HER2-negative and HER2-positive [21, 27–30]. Some authors suggest that the prevalence of *HER2* mutations changes according to certain histological subtypes in breast cancer [21, 27, 31].

Recently, data from preclinical and clinical studies have attributed somatic mutations in *HER2*, a role in the constitutive expression [31–33] or differential regulation of HER2 that leads to resistance (primary or acquired) to anti-HER2 therapy and endocrine therapy [4, 6, 10, 34–36]. Such mutations therefore undermine the clinical benefits of HER2-targeted treatment in HER2-positive breast cancer patients. Besides, different mutations in *HER2* have been found in several tumors although their role in tumorigenesis is not fully understood. To assess the possible clinical implications of HER2 mutations in HER2-positive breast cancer patients, we here review the spectrum of single nucleotide polymorphisms (SNPs) produced in the *HER2* gene. Our working hypothesis was that recurrent mutations in specific HER2 domains in these patients could be good biomarkers of the efficacy of anti-HER2 therapy.

2. Methods

To identify mutations in the *HER2* gene in HER2-positive breast cancer patients, two databases were searched: cBioPortal [37] and COSMIC [38]. These websites provide information regarding the largest number of studies and *HER2* mutations across different cancer types. To identify mutations reoccurring in HER2-positive breast cancer, the following keywords were used: HER2+ BREAST CANCER, ER-HER2+ BREAST CANCER, and ER-PR-HER2+ BREAST CANCER. In both databases, mutations were observed at similar frequencies. To obtain functional data for the different mutations, we also undertook a PubMed [39] search for articles written in English using the keywords: BREAST CANCER, CANCER RISK, HER2/ERBB2, HER2 POSITIVE, HER2-TYROSINE KINASE DOMAIN, HER2, HER2-TRANSMEMBRANE DOMAIN, HER2-EXTRACELLULAR DOMAIN, and HER2 MUTATIONS.

2.1. Mutations in HER2 Gene in Different Breast Cancer Histologies. Mutations in the ERBB2 receptor described in this study according to the tumor type were found in invasive lobular carcinoma (ILC), invasive ductal carcinoma (IDC), and mixed ductal and lobular carcinoma (MDLC) (Table 2). There is variability in the distribution of the different mutations depending on the specific histology of the breast cancer type. Seven of the eleven mutations were present in both types of carcinomas or even in mixed carcinomas (MDLC); however, some of these mutations are mainly found in IDC or others in ILC (Table 2). Thus, mutations located mainly in IDC were D769H, V842I, K753E, R678Q, and S310F I655V. In the other side, mutations more prevalent in ILC were L755S, V777L, D769Y, and S310Y. Previous studies suggest that *HER2* mutations
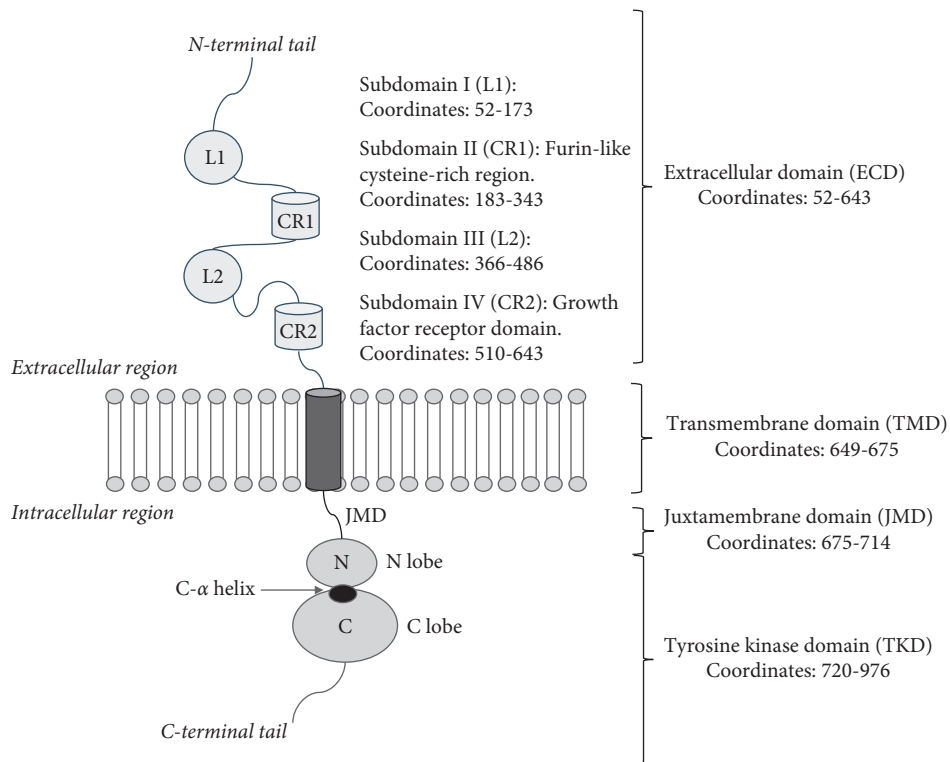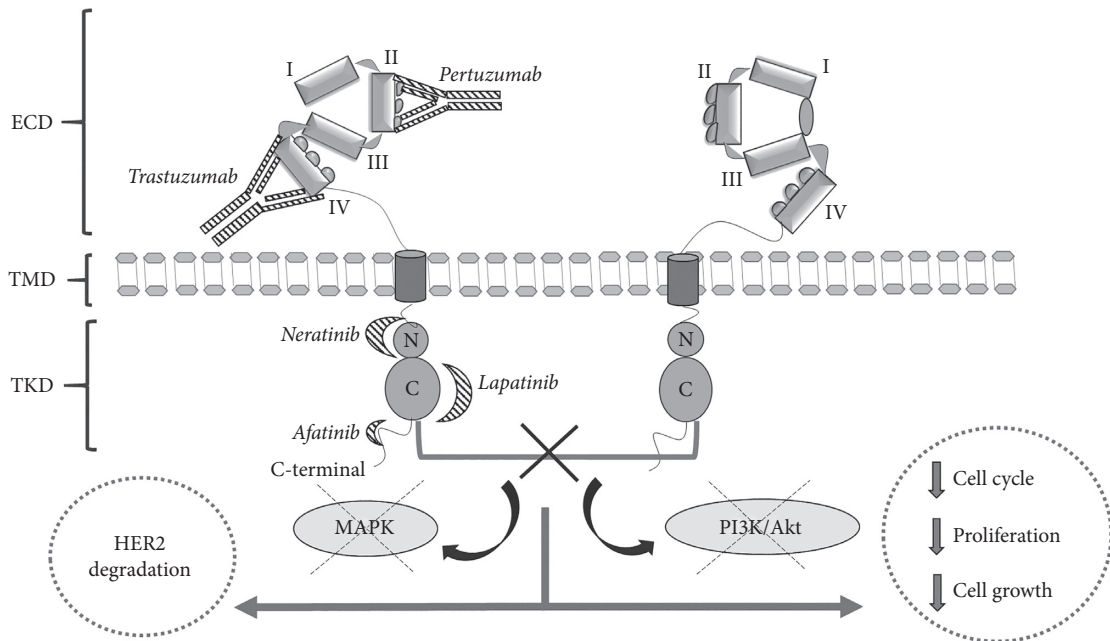
FIGURE 1: Structural domains of HER2 protein.

FIGURE 2: The mechanism of action of different drugs (italics and striped) on HER receptor signaling pathways. HER: human epidermal growth factor receptor; MAPK: mitogen-activated protein kinase; PI3K: phosphatidylinositol 3-kinase; Akt: serine/threonine kinase Akt, also known as PKB (protein kinase B); ECD: extracellular domain; TMD: transmembrane domain; TKD: tyrosine kinase domain.

are enriched in certain histological subtypes, as example, some authors have indicated that invasive lobular breast cancer (ILC), which composes about 15% of estrogen re- ceptor- (ER-) positive subtype, the prevalence of *HER2* mutations is higher (cBioPortal-21, 27, 56-ILC). No

quantitative analysis of the presence of specific mutations according to tumor type has been performed in this study, but the *HER2* mutations described here located in IDC and ILC are in agreement with other studies [27, 31, 56, 57]. Interestingly, *in silico* analysis suggests that some *HER2*

TABLE 1: Current therapeutic approaches targeting HER2 signaling [7, 22–25].

Drug	Molecular target	Molecular mechanism	Treatment options
Lapatinib	TKD ⟶ HER2 and HER1 ATP mechanism of action binding sites	Reversible inhibitor of HER1 and HER2 trans- and autophosphorylation	With metastasis: +capecitabine or letrozole +trastuzumab
Neratinib	TKD ⟶ HER1, HER2, and HER4 ATP binding sites	Irreversible inhibitor of HER1, HER2, and HER4 trans- and autophosphorylation	Adjuvant after trastuzumab treatment
Trastuzumab	Subdomain IV of HER2 ECD	Inhibitor of HER2 homodimerization	First-line anti-HER2 treatment
Ado-trastuzumab emtansine (T-DM1)	Subdomain IV of HER2 ECD	Inhibitor of HER2 homodimerization, cytotoxic action of emtansine	Specific cases after anti-HER2 treatment with trastuzumab
Pertuzumab	Subdomain II of HER2 ECD	Inhibitor of HER2 heterodimerization	Dual therapy: anti-HER2 with trastuzumab + docetaxel/paclitaxel or + capecitabine/vinorelbine
Afatinib	TKD: HER1, HER2, HER4 ATP binding sites	Irreversible inhibitor of HER1, HER2, and HER4 trans- and autophosphorylation	Under research: used as monotherapy in patients with HER2-positive breast cancer showing progression despite trastuzumab treatment. Pending FDA approval

TKD: tyrosine kinase domain; ECD: extracellular domain; HER2: human epidermal growth factor receptor 2.

mutations are enriched in primary ILC and their detection represents an actionable strategy with the potential to improve patient outcomes with estrogen receptor-positive, ERBB2 nonamplified primary lobular [27]. Overall, more quantitative studies are needed for the identification of co-occurring and mutually exclusive *HER2* mutations according to histology subtype in order to identify patient that could potentially be targeted with HER2-directed therapies.

2.2. Mutations in the Tyrosine Kinase Domain. Most mutations in the *HER2* gene have been detected in exons 19 and 20 of the tyrosine kinase (TK) domain, at the C-α helix position of the protein [34] (Table 2). Several authors propose that mutations in this domain could be an alternative mechanism to HER2 activation and affect sensitivity to anti-HER2 therapy, as an acquired resistance mechanism to this form of therapy. The TKD mutations described to date in HER2+ breast cancer promote the activation of the functionality of the protein and increase the oncogenicity of HER2, besides inducing the phosphorylation of other cell signaling proteins [28, 34] (Table 2). This is because this domain contains the ATP binding site and its mutations are related to the enhanced phosphorylation of receptors HER2, HER3, and HER1, which causes receptor HER2 dimerization along with protein ERK (extracellular signal-regulated kinase) and AKT phosphorylation, with consequent activation of the PI3K/Akt and MAPK pathways, finally enhancing cell proliferation and angiogenesis (Figure 2). The binding site of ATP with the receptor protein forms a conformational structure with other important structures such as phosphate activation and binding loops, which could be affected by such modifications. Missense substitutions usually occur at the C-α helix, which is essential for HER2 protein activation. These alterations can promote tumorigenesis and phosphorylation

of signaling proteins such as phospholipases γC1 and Cγ (PLCγ) MAPK. Many of these activating mutations have proved resistant to anti-HER2, such as those found at codons 755 or 798 [34].

Most authors have described the appearance of both intrinsic and acquired resistance to trastuzumab therapy in mutations L755S, V777L, D769Y, and K753E [32, 40, 42, 44, 45]. As these mutations are not located close to the drug's binding target, it seems that rather than blocking receptor binding of the drug, they affect resistance to its effects by increasing kinase activity and activation of the protein's oncogenic signaling pathways, independently of drug binding. All these mutations as well as D769H share the feature of sensitivity to the actions of the irreversible TK inhibitor, neratinib [28, 30, 35, 41, 42, 45]. This could be explained by the greater strength of interactions produced between this drug and the ATP-binding site. This response offers a good treatment option for patients who may have developed resistance to first-line treatments for HER2+ breast cancer. Most authors agree that resistance to lapatinib, both intrinsic and acquired, appears in L755S, D769Y, V842I, and K753E [41, 42, 44]. This indicates the importance of the electrostatic interactions that occur at the ATP binding site close to these residues. Moreover, depending on the changes produced by the amino acid substitutions, a protein conformation may arise that promotes either the active state of HER2's kinase domain impairing proper drug binding or this binding increases sensitivity toward the drug.

The L755S mutation is the most common in *HER2* gene [41] and is considered a hotspot mutation [58]. The protein's codon 755 seems to be strongly involved in activating HER2 receptor kinase, which leads to the potentiated activity of the PI3K/Akt and MAPK signaling pathways, giving rise to enhanced cell proliferation and angiogenesis. In preclinical trials, this mutation has been associated with resistance to lapatinib treatment through reactivation of HER2 signaling

Table 2: Main features and pharmacological implications of the HER2 gene SNPs reviewed in HER2-positive breast cancer patients. ILC: invasive lobular carcinoma; IDC: invasive ductal carcinoma; MDC: mixed ductal and lobular carcinoma. These mutations are found also in HER2-negative breast cancer [28, 29, 32, 34, 77].

Mutation	Exon	Tumor type	Protein domain	Mutation impact	Pharmacological implications	Study design	References
L755S	19	ILC IDC MDC	TKD, C-α helix	Activation	Trastuzumab/lapatinib resistance Neratinib/afatinib sensitivity	Breast cancer HER2+ patients, *in vitro* studies	[40, 41]
					Lapatinib resistance	Breast cancer HER2+ patients, *in vitro* studies	[42, 43]
					Trastuzumab resistance Afatinib/neratinib sensitivity	MANO method and xenograft	[44]
					Afatinib/neratinib sensitivity	MANO method and xenograft	[41, 44]
V777L	20	ILC IDC	TKD, C-α helix, C-terminal tail	Activation	Trastuzumab resistance Lapatinib/neratinib sensitivity	Breast cancer HER2+ patients	[45]
					Trastuzumab resistance Lapatinib/neratinib/afatinib sensitivity	MANO method and xenograft	[44]
					Trastuzumab + lapatinib sensitivity	Breast cancer HER2+ patient	[31]
					Neratinib sensitivity	Breast cancer HER2+ patient	[35]
D769Y	19	ILC IDC	TKD, C-α helix	Activation	Neratinib sensitivity Trastuzumab/lapatinib resistance	Xenograft study	[42]
					Trastuzumab resistance Afatinib/lapatinib/neratinib sensitivity	MANO method and xenograft	[44]
D769H	19	IDC	TKD, C-α helix	Activation	Neratinib sensitivity	Breast cancer HER2+ patient	[28]
					Trastuzumab/pertuzumab sensitivity	Breast cancer HER2+ patient	[46]
					Trastuzumab/afatinib/ lapatinib/neratinib sensitivity	MANO method and xenograft	[44]
I767M	19	IDC ILC MDLC	TKD, C-α helix	Inconclusive	Trastuzumab/lapatinib/ afatinib/neratinib sensitivity	*In vitro* breast cell cultures; MANO method; xenotransplant; breast cancer HER2+ patients	[44, 47]
V842I	21	IDC ILC	TKD, c-loop	Activation	Lapatinib/trastuzumab resistance	MANO method and xenograft	[44]
K753E	18	IDC	TKD, C-α helix	Likely neutral	Lapatinib/trastuzumab resistance Neratinib sensitivity	Breast cancer HER2+ tumors	[32, 41]
R678Q	17	IDC	JMD	Activation	Trastuzumab/lapatinib/ afatinib/ Neratinib sensitivity	MANO method and xenograft	[44]
I655V	16	IDC	TMD	Activation	Trastuzumab sensitivity		[48]
					No correlation with trastuzumab efficacy		[49–51]
					Trastuzumab resistance	Breast cancer HER2+ patients	[52]
					No correlation with trastuzumab-induced cardiotoxicity		[53]
					Correlation with trastuzumab-induced cardiotoxicity		[50]
S310F	8	IDC ILC	ECD, subdomain II, furin-like domain CR1	Activation	Neratinib/trastuzumab sensitivity	Breast cancer HER2+ patients	[33, 54]
					Trastuzumab/lapatinib/ afatinib/neratinib sensitivity	MANO method and xenograft	[44]
S310Y	8	ILC IDC MDLC	ECD, subdomain II, furin-like domain CR1	Activation	Neratinib/trastuzumab sensitivity	Breast cancer HER2+ patients	[33, 55]
					Trastuzumab/lapatinib/ afatinib/neratinib sensitivity	MANO method and xenograft	[44]

in HER2+ breast cancer models in which the gene is overexpressed [43, 44]. In *in vitro* models, it was observed that cells with this mutation were resistant to treatment with lapatinib + trastuzumab, but also to trastuzumab + pertuzumab treatment [32, 40, 41]. As this mutation induces resistance to trastuzumab alone or in combination with pertuzumab, despite its location far from the drugs' binding sites on the receptor, it could be that kinase activity is so enhanced that it is able to continue signaling despite the nondimerization of the receptor after the binding of these drugs [40, 41, 44, 59]. Resistance to lapatinib can be explained by the fact that Leu 755 participates in hydrophobic interactions with the C-α helix of the TKD in the active state of HER2, while in the inactive form, L755 is found far from this helix [43]. The L755S polymorphism induces the appearance of polar interactions that stabilize the active form; this would help explain resistance to lapatinib, which only binds to the inactive conformation of HER2 [43]. This resistance could be addressed with irreversible HER1 and HER2 inhibitors such as neratinib, which has proven effective in patients with this mutation [41]. In effect, *in vitro* studies have shown the sensitivity of cells with the L755S mutation to afatinib plus neratinib [41, 44]. Besides intrinsic resistance, mutation L755S has been associated with resistance acquired to trastuzumab therapy in breast cancer. It appears in 7.59% of patients receiving prior trastuzumab treatment. Further, it has been reported to occur in 3 out of every 18 patients with metastasis but not those with primary tumors [41].

Mutation V777L is also considered hotspot [58]. Residue V777L, located in exon 20 (at the C-terminal tail of the C-α helix), is involved in TK activity. This activating mutation promotes the TK activity of HER2, increasing the phosphorylation of signaling proteins such as HER2, HER3, EGFR, and ERK, and the transformation of breast epithelial cells [29, 33, 40, 45, 60]. This mutation causes transcriptional activation in most tumors affected by this mutation, which usually occurs independently of *HER2* gene activation [60]. In effect, cases have been described in breast cancer cell lines in which increased endogenous expression levels of HER2 V777L activated signal transduction pathways, but this did not significantly increase tumor growth [61]. The effects of V777L seem enhanced by mutations in the PIK3CA gene given that, in the presence of mutation PIK3CA E545K, V777L gives rise to enhanced interaction between p58 and HER3. This suggests that reverse mutations of the *HER2* gene could require other genetic alterations to promote cellular transformation and enhance interactions between signaling partners [31]. This mutation has been associated with the intrinsic development of trastuzumab resistance [45]. Although the mutation has been associated in some preclinical studies with a diminished response to lapatinib, afatinib, and neratinib, several studies have shown reduced tumor growth and signaling activity in tumors with the V777L mutation treated with lapatinib [44, 45]. A response has been observed to combined treatment with neratinib and other drugs in patients with ER + V777L breast carcinoma [35]. No cases relating this mutation to the response to pertuzumab have been described. Considering that this last drug, as does trastuzumab, binds to the extracellular domain of the protein and that resistance to trastuzumab has been described, we would expect pertuzumab to neither elicit a good response in patients with this mutation. As occurs with the L755S mutation, *HER2* V777L shows strong activation of the receptor's kinase that could preserve its signaling activity even with trastuzumab and pertuzumab bound to the extracellular domain of the protein.

Interestingly, V777L and L755S mutants have been characterized using molecular dynamics simulations and *in vitro* studies in Ba/F3 cells expressing these mutants, showing that these mutants have a larger binding pocket volumes and therefore are more sensitive to tyrosine kinase inhibitors (TKIs) of quinazoline (afatinib and poziotinib) and indole (osimertinib and nazartinib) groups. Furthermore, in preclinical models, poziotinib upregulates HER2 cell surface expression and potentiates the activity of T-DM1, inducing a complete tumor regression with combination treatment [62]. The authors of this study suggest that poziotinib in combination with T-DMI could be a good candidate treatment for not only non-small cell lung cancer; in fact, one ongoing trial in phase II is studying the efficacy of poziotinib in metastatic breast cancer harboring *HER2* mutations [21, 62]. Overall, more clinical studies are needed to test the efficacy of poziotinib in combination with T-DMI in breast cancer to rule out differences in tumor type-specific sensitivities to the same pharmacological product. In SUMMIT trial, neratinib was most effective in breast cancer patients, with patients containing L755S and V777L [33], but the same mutations were associated with resistance in other cancer types, suggesting that more research is needed to identify the mechanism involved in tumor-type-specific sensitivities.

Recently, using isogenic knock-in *HER2* mutations in ER + MCF7 cells and xenografts, two activating *HER2* mutations located in the kinase domain (L755S and V777L) emerged as resistance to anti-ER therapy progression [35]. These findings are corroborated by other authors, and the same mutations have been identified in metastatic biopsies of eight patients with ER + metastatic breast cancer (MBC), as mutations that were acquired under the selective pressure of ER-directed therapy such as aromatase inhibitors [36]. The same authors demonstrated that the resistance to ER-directed therapy was overcome by combining fulvestrant with the irreversible HER2 kinase inhibitor neratinib. These data suggest that the prevalence of *HER2* mutations might increase in metastatic ER+ breast cancer treated with anti-ER therapy, and these mutations are a distinct mechanism of acquired resistance to ER-directed therapy in metastatic breast cancer that could be solved by the treatment with an irreversible HER2 inhibitor. Overall, these data suggest that patients with ER+/HER2 mutations would benefit from HER2-targeted therapies in combination with hormonal therapy. If ongoing clinical trials confirm these results, new approaches could be adopted in order to promote a better response in patients with ER + MBC, and one of these strategies could be to identify HER2-mutant-resistant clones to ER-directed therapy [36].

Mutation V842I has been detected in various types of tumor tissue. This is also an activating mutation associated with *HER2* gene amplification and increased phosphorylation of different signaling proteins [28] and also represents a hotspot in *HER2* [58].

The effects of V842I on the response to anti-HER2 therapy in patients with HER2+ breast cancer have not been yet explored. Some *in vitro* studies indicate the resistance to trastuzumab and lapatinib of cell lines with this mutation [44]. This mutation is the most common mutation in colorectal cancers, and *in vitro* studies have shown that this mutant was not sensitive to neratinib [62]. However, given its recurrent expression in different tumor tissues and its association with amplification of the gene, studies are warranted to clarify its impact on the receptor's kinase activity.

The nonsynonymous mutations D769Y and D769H are among the most frequent somatic mutations of the *HER2* gene. They are located in exon 19, at position 769 of the TK domain, which is important for ATP-HER2 binding [29]. Both mutations have been characterized as activators in mammary epithelium cell lines, and *in vivo* studies have revealed neratinib as effective at blocking tumor growth in HER2+ breast carcinomas with these mutations [28, 42].

Cases have been described of xenografts acquiring the D769Y mutation following treatment with trastuzumab, along with their subsequent resistance to trastuzumab and lapatinib, suggesting its possible role in acquired resistance to anti-HER2 therapy [42]. In mutation D769Y, the change from aspartic acid to tyrosine could lead to changes in electrostatic interactions, due to the substitution of a negatively charged acid side chain at physiological pH with the capacity to form hydrogen bridges and bind phosphate groups. As this mutation occurs at an important position for ATP binding to the receptor, this change could benefit this binding and thus diminish the impacts of lapatinib and neratinib therapy, whose mechanism of action is to impair this binding of ATP to HER2 [42]. The D769Y mutation promotes the phosphorylation of HER2, EGFR, HER3, and ERK and transformation of mammary epithelial cells. Cell lines with this mutation display sensitivity to neratinib, in smaller measure to lapatinib and resistance to trastuzumab [42], although Nagano et al. recently described sensitivity to lapatinib and afatinib in *in vitro* studies [44]. Some authors report that loss of the acid side chain or addition of an aromatic ring to amino acid 769 could increase HER2's TK activity due to dimeric interactions between the kinase domains of HER2 and HER3. Mutations D769H/Y may enhance hydrophobic contacts and heterodimerization of HER2. Besides, the D769H alteration could lead to activation within the HER2 monomer, adding hydrogen bonds to its own activation A-loop [46, 54].

Mutation K753E leads to a shift in charge of the amino acid's side chain, which goes from being basic to acidic, thus possibly affecting the electrostatic interactions of the protein. Several authors have related this mutation with lapatinib resistance, and this could be attributed to its close proximity with the L755S mutation which confers resistance to this drug [32, 41]. Recently, the effect of this mutation has been observed in cell lines overexpressing HER2 K753E. In HER2 K753E mutant cells resistant to lapatinib, a greater affinity of the drug for the HER2 protein was observed compared to wild-type cells and other variants. This reveals that resistance to this drug is unrelated to a lack of binding to its target [63]. It has also been related to resistance to trastuzumab and appears in 2 out of every 18 patients with metastasis [32]. While cell lines that show this mutation are resistant to lapatinib, they are sensitive to neratinib, which could benefit patients developing resistance to trastuzumab therapy [41].

Following trastuzumab therapy, the appearance of K753E and L755S mutants could suggest their potential role as drivers of developing trastuzumab resistance during HER2+ tumor progression [32].

Mutation I767M is a hotspot in gene *HER2* [58] identified in patients with HER2+ breast cancer [54]. Its expression has been examined *in vitro* in HER2-overexpressing mammary cell lines and in HER2-negative cultures. In the former cells, the presence of this mutation along with mutations in the genes *PIK3CA* and *TP53* conferred a significant growth benefit over cells with the wild-type HER2 gene. Further, both the mutant and wild-type protein featured similar AKT and MAPK signaling levels, although the AKT pathway remained active over time for longer in the cells expressing HER2 I767M [47]. *In vitro* studies conducted by Nagano et al. [44] indicate the sensitivity of I767M to therapy with both TK inhibitors (lapatinib, neratinib, and afatinib) and the monoclonal antibody trastuzumab.

According to the data from COSMIC and cBioPortal, while other mutations in this kinase domain have been described (i.e., V797A, D808E, D873G, and M889I), there are still no data regarding their role in HER2+ breast cancer.

2.3. Mutations in the Juxtamembrane Domain.

The juxtamembrane domain, containing 39 amino acids (Figure 1), is involved in receptor dimerization and stability. Several authors have described reoccurring mutations in this domain with a functional activating effect in different cancer types [10]. However, these studies do not specify if these mutations occur in patients with HER2-positive breast cancer. In our search of mutations in the COSMIC and cBioPortal databases, we found two mutations, R678Q and V697L, present in HER2-positive breast carcinoma. *In vitro* studies indicate that R678Q is an activating mutation that confers sensitivity towards treatment with trastuzumab, lapatinib, afatinib, and neratinib (Table 2) [10, 33, 44] and has been classified as a hotspot [64]. No functional data exist for mutation V697L, but it has been described as a mutational hotspot and data available for other cancer types suggest its oncogenic effect.

2.4. Mutations in the Transmembrane Domain.

The transmembrane domain of receptor HER2 (aa 649–675, Figure 1) plays an active role in its dimerization with the consequent activation of kinase activity and promotion of the signaling pathways responsible for tumor cell growth. Recently, recurrent mutations have been identified with an activating

effect in different cancers [10]. However, the data sources COSMIC and cBioPortal reveal that no mutations in this domain occur in HER2-positive breast cancer. In the present study, we identified only one mutation, I655V (Table 2), in HER2-positive patients, which, according to Fleishman et al. [65], involves an altered receptor conformation that renders it a constitutively activated state, promoting the homo-dimerization and autophosphorylation of HER2 and activation of the TK domain [65]. Singla et al. [49] found, among patients in Indian hospitals, a positive significant association between *HER2* I655V and the susceptibility of developing breast cancer, while other authors have detected negative correlation when examining patients in Brazil [66]. A meta-analysis conducted in 2019 [67] revealed the impacts of ethnicity on the association between mutation *HER2* I655V and breast cancer risk, observing positive correlation in Asia and Africa but not the other continents.

The relationship between this mutation and the response to trastuzumab-containing chemotherapy in HER2-positive breast cancer patients has been examined, yet results have been contradictory. In some studies, disease-free survival (DFS) and delayed DFS (DDFS) were improved in patients with this mutation and the genotypes *HER2* Val/Val or Val/Ile compared to the genotype Ile/Ile [48]. On the contrary, Furrer et al. [52] noted a worse response to trastuzumab-containing chemotherapy, while other studies have found no correlation [49–51]. These results preclude establishing a clear relationship between this polymorphism and the development of resistance to treatment with trastuzumab. The effect of trastuzumab and other antibodies may be limited in tumors that show mutations in the TMD, as HER2 dimerization seems stable despite trastuzumab binding to its extracellular domain. Neratinib, which binds to the HER2 receptor's kinase domain and inhibits its phosphorylation and activity, can exert antitumor effects irrespective of the domain affected by mutations and has an impact on this *HER2* I655V mutation in different lung cancer cell lines [68].

Something similar occurs with the risk of cardiotoxicity induced by trastuzumab. Despite initial proposals that being a carrier of the mutant allele, genotypes *HER2* Val/Val or Val/Ile, was a possible predictor of this adverse effect of the drug [50], this relation could not be later confirmed [53]. Some authors have suggested that carrying this mutation only affects the survival of patients with breast cancer who overexpress the *HER2* gene [48]. To date, the possible effect of this mutation on the actions of other HER2-target drugs such as pertuzumab, neratinib, afatinib, or lapatinib has not been addressed.

2.5. Mutations in the Extracellular Domain.

The extracellular domain is composed of four subdomains involved in dimerization of the receptor and thus in its activation. Several mutations in the ECD domain have been described in patients with HER2-positive breast cancer both in PubMed and the databases COSMIC and cBioPortal, as described below. The most common mutations in the ECD of the HER2 receptor, S310F and S310Y, corresponding to the gene hotspot (Table 2) [64], have been related to the increased

dimerization of the receptor, kinase activation, and malignant cell transformation. Both mutations appear to have a homologous effect. Of the two, S310F has been most studied in different tumor tissues (both HER2-positive breast and HER2-negative lobular breast, lung, colorectal, ovarian, bladder, micropapillary urothelial, and endometrial) [29, 33, 54, 68–70] while S310Y has been more commonly associated with pulmonary adenocarcinoma while it has been also found in HER2-positive and HER2-negative breast cancer [29, 33, 55, 71]. The fact that mutations in this position are present in different cancers suggests it could be an oncogenic mutation [72]. They are therefore mutations that activate HER2 protein via elevated phosphorylation of the C-terminal tail, as is the case of mutation S310F/Y, or inducing covalent dimerization sustained by intermolecular disulfide bridges [73], as in the case of mutations G309E/A, only described in HER2-negative breast cancer and other cancers [28, 73]. In the presence of an S310F/Y mutation, it has been noted that protein HER2 seems more sensitive to anti-HER2 therapy containing neratinib and possibly trastuzumab in patients with HER2+ breast cancer [33, 54, 55]. Accordingly, in cells featuring ECD mutations, trastuzumab may bind to this region and prevent homodimerization and activation of the receptor. However, because of the constant activation of the TKD in tumors with mutations at this domain, the antiproliferative effects of monoclonal antibodies may be limited despite inhibiting dimerization [74]. The effects of these mutations on the response to pertuzumab and trastuzumab have been investigated recently using *in vitro* 5637 culture cells and single-molecule interaction analyses using TIRF microscopy [74]. The overexpressed S310F as well as G309A, G309E, and S310Y HER2 mutants reacted to trastuzumab, but S310F mutant did not react to pertuzumab along with S310Y or G309E mutants. Thereafter, authors tested the effects of trastuzumab and pertuzumab using both wild-type HER2 and S310F mutant. In this case, trastuzumab did not inhibit the activation of the HER2 receptor, suggesting that the S310F HER2 mutant did not form homodimers or heterodimers with wild-type HER2. Because pertuzumab did not inhibit the phosphorylation of HER2 while it bound to wild-type HER2, EGFR-mediated phosphorylation is expected to occur on the S310F mutant; therefore, trastuzumab in combination with pertuzumab is not effective [74]. This residue is located close to one of the key residues, K311, for receptor-antibody binding whose replacement with alanine, via targeted mutagenesis, leads to a drastic reduction in the response to this drug in cells expressing the mutation. Amino acid substitutions in these residues could provoke changes in electrostatic interactions or even give rise to a stearic impediment possibly affecting pertuzumab binding to the HER2 receptor [69].

Other less frequent mutations in the ECD have been described, such as R190Q, P523S, and Q548R, in patients with breast cancer without specifying HER2 amplification, in which no relationship has been found between the mutations and prognosis [75]. Even rarer are L313I and R456C, observed in two patients with HER2+ breast cancer effectively treated with neratinib [42].

The COSMIC and cBioPortal databases also describe other mutations in this domain about which there are no published data for HER2-positive breast cancer: A37T, P232S, D277H (also described in bladder cancer, enhances its activation together with S310F, de Martino et al. [76]), T297I, E405D, and H470Q.

2.6. Beyond HER2+ Breast Cancer: Lessons from Clinical Data from the Use of HER2-Directed Therapy against HER2 Mutant Cancers. Around sixteen clinical trials investigating the efficacy of HER2-directed therapy in HER2 mutant cancers are currently active [21]. Four phase II studies are studying the efficacy of different pharmacological products (afatinib, neratinib plus trastuzumab, poziotinib, and pyrotinib) in different types of metastatic HER2 non-amplified but with HER2 mutant breast cancer. There are a relatively large number of pharmacological approaches for breast cancer carrying HER2 mutants. In part, we have reached this situation because activating mutations for *HER2* have been shown to be largely dependent on tumor histology and have shown different clinical responses. Some mutations are sensitive in a specific type of cancer and in others could be associated with resistance, suggesting that there may be other mechanisms specific with the tumor that requires further research.

The focus of this review is to assess the possible clinical implications of *HER2* mutations in HER2-positive breast cancer patients; in this study, we have described that the most prevalent mutations found in *HER2* gene in HER2-positive breast cancer (Table 2) are present also in HER2-negative breast cancer [28, 29, 32, 34, 57]. We would like to address, using clinical data available, if HER2-negative patients with *HER2* somatic mutations are potentially good candidates for HER2-directed therapy. The clinical data available have been reviewed by Cocco et al. [21] and are summarized in Table 3. The first patient diagnosed with triple-negative breast cancer, carrying two HER mutations (V777L and S310F), respond to lapatinib and trastuzumab-based therapies during 6 months. A second case diagnosed with ER + HER-negative breast cancer, carrying a HER2 S310F mutation, was treated during 12 months with the combination of trastuzumab, pertuzumab, and fulvestrant. An additional case with ER+, HER2-negative metastatic breast cancer with HER L755S mutation was treated with neratinib monotherapy experiencing improvement in symptoms and tumor markers. Another case described a *HER2* (D769H) mutant with metastatic HER2-negative breast who achieved a partial response with trastuzumab, pertuzumab, and chemotherapy (Table 3). These clinical data are in agreement with the pharmacological profile of the SNPs of HER2 reviewed in this study (Figure 3). In the phase II MutHER trial, the activity of neratinib in HER2 mutant nonamplified metastatic breast cancer was investigated (Table 3); the patients obtained clinical benefit over 24 months [77]. A case report was a HER2-negative breast cancer patient with two detected mutations in ERBB2 (S310F and D769Y mutations) who benefited from lapatinib combined with endocrine therapies [78]. Based on clinical data available for HER2-negative breast cancer patients (Table 3), functional activating HER2 mutations, V777L, L755S, S310F, D769H/Y, and V842I, may similarly confer sensitivity to HER2-directed pharmacological products. Furthermore, a high number of HER2-mutant tumors are also ER+, and as discussed before, the most effective treatment will be combining fulvestrant with the irreversible inhibitor neratinib [21, 35]. Overall, HER2-negative breast cancer patients carrying the above mutations can benefit from HER2-targeted therapy; this is in agreement with data previously published by other authors [32, 78].

2.7. Conclusions and Future Perspectives. Research and clinical studies have shown that HER2 overexpression/ amplification is associated with poor survival in breast cancer patients. Further, *in vitro* and *in vivo* studies indicate that the presence of somatic HER2 mutations could influence the clinical outcome of HER2-positive patients under currently approved treatments (Table 1). New findings in breast cancer cells suggest that HER2 could interact physically with PMCA2 and the cannabinoid receptor CB_2R. Hence, targeting these heteromers could be a new therapeutic option and prognostic tool in HER2-positive breast cancer. Recently, *in vitro* and *in vivo* studies in metastatic ER + tumors suggest that some HER2 mutations emerge as a mechanism of acquired resistance to endocrine therapy opening new options of treatments in patients with ER + MBC.

In this review, we identify the more prevalent somatic HER2 SNP mutations appearing in HER2-positive breast cancer patients and summarize their possible implications for current HER2-targeted therapy (Figure 4). We found that somatic HER2 mutations occur in low frequency in HER2-positive breast cancer patients. In total, 11 somatic mutations have been identified, and according to information available from *in vitro* and *in vivo* studies, 9/11 are classified as oncogenic and hotspot (see Table 2), and several authors have identified the presence of these mutations also in HER2-negative breast cancer patients. For two mutations, I767M and K753E, there is insufficient information so far to classify them as oncogenic and/or hotspot. In HER2-positive tumors, the TKD harbored the higher number of somatic mutations (7/11), contrasting with the low number of mutations found in the extracellular and transmembrane domains. The relevance of some mutations identified in this study requires further investigation.

For the reviewed somatic HER2 mutations, no sensitivity or resistance data are available for pertuzumab, with the exception of mutation D769H. For some mutations, available data are inconclusive requiring more functional studies. HER2-positive patients carrying S310F, S310Y, R678Q, D769H, I767M, or V777L emerged as potentially good candidates for HER2-targeted therapy and could have a favorable outcome because of sensitivity to current pharmacological treatments with the exception of inconclusive data for the impacts of trastuzumab in V777L (Figure 3). Patients with L755S or D769Y might also benefit from neratinib or afatinib treatment. In contrast, patients with the

TABLE 3: Clinical response of HER2 mutant breast tumors to anti-HER2-based therapy.

No. patients	Type of breast cancer	HER2 mutation	Pharmacological treatment	Outcome	Reference
1	Triple-negative	V777L S310F	Lapatinib, trastuzumab	Improvement during 6 months	Reviewed in 21
1	ER+/HER-negative	S310F	Trastuzumab, pertuzumab fulvestrant	Improvement during 12 months	Reviewed in 21
1	ER+/HER-negative	L755S	Neratinib	Improvement during 12 months	Reviewed in 21
1	Metastatic HER2-negative	D769H	Trastuzumab, pertuzumab chemotherapy	Partial response	Reviewed in 21
1	HER2-negative	S310F/V842I	Neratinib	Benefit	[77]
6	HER2-negative	L755S	Neratinib	Benefit	[77]
1	HER2-negative	D769H	Neratinib	Benefit	[77]
1	HER2-negative	p.L755_T759del	Afatinib, trastuzumab	Response	[78]
1	HER2-negative	S310F and D769Y	Lapatinib and endocrine therapy	Response	[78]

	Tyrosine kinase domain							Juxtamembrane domain	Transmembrane domain	Extracellular domain
	L755S	V777L	D769Y	D769H	I767M	V842I	K753E	R678Q	I655V	S310F/S310Y
Trastuzumab	Resistant	Inconclusive	Resistant	Sensitive	Sensitive	Resistant	Resistant	Sensitive	Inconclusive	Sensitive
Pertuzumab				Sensitive						
Lapatinib	Resistant	Sensitive	Inconclusive	Sensitive	Sensitive	Resistant	Resistant	Sensitive		Sensitive
Neratinib	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive		Sensitive	Sensitive		Sensitive
Afatinib	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive			Sensitive		Sensitive

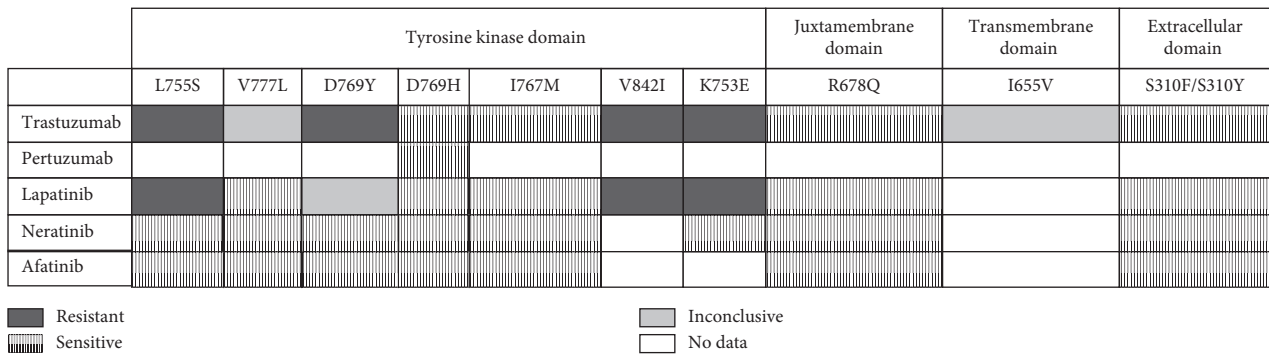Legend: ■ Resistant · ▥ Sensitive · ▨ Inconclusive · □ No data

FIGURE 3: Pharmacological impacts of the SNPs reviewed in this study. The sensitivity of HER2 mutants to different drugs used as anti-HER2 therapy is shown. The pharmacological products have different levels of activity against mutant HER2+ proteins *in vitro*. When data from *in vivo* studies (xenotransplant and/or breast cancer patients) were available, they were considered for the analysis. Furthermore, some mutants that have been described to be sensitive to specific inhibitors in preclinical analyses were instead found to be resistant to the same drugs; in this case, we have indicated this information as inconclusive data.
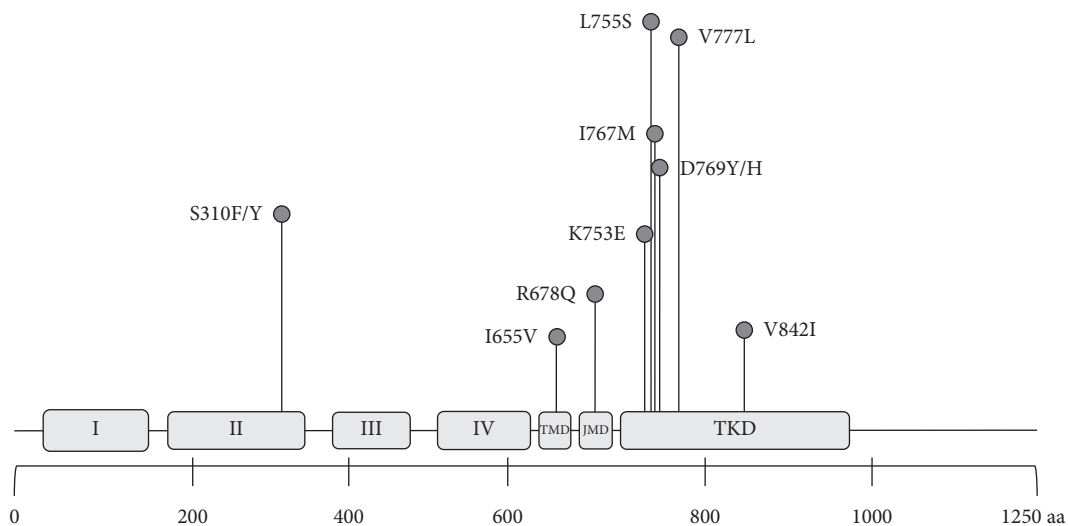
FIGURE 4: Schematic diagram of HER2 protein with the locations of the SNPs reviewed in this study found in HER2-positive breast cancer patients. Domains I, II, III, and IV belong to the extracellular domain (ECD); TMD: transmembrane domain; JMD: juxtamembrane domain; TKD: tyrosine kinase domain.

somatic mutations L755S, V842I, K753I, or D769Y do not seem to benefit from trastuzumab. Similar negative results have been observed for lapatinib in patients carrying the L755S, V842I, and K753I mutations. L755S and V777L mutations emerge as a distinct mechanism of acquired resistance to anti-ER therapies in ER+ metastatic breast cancer that was overcome by combining fulvestrant with the irreversible inhibitor neratinib. Furthermore, patients with metastatic breast cancer HER2+ with L755S and V777L could benefit of treatment with a new TKI, poziotinib, that is in phase II of clinical trials. Clinical studies suggest that HER2-negative breast cancer patients carrying the *HER2* mutations reviewed here can benefit from HER2-targeted therapy. In future studies, different combinations of mutations in patients and their treatment with different combinations of drugs need to be considered.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

References

[1] J. A. Merino Bonilla, M. Torres Tabanera, and L. H. Ros Mendoza, "El cáncer de mama en el siglo XXI: de la detección precoz a los nuevos tratamientos," *Radiología*, vol. 59, no. 5, pp. 368–379, 2017.

[2] S. Jeibouei, M. E. Akbari, A. Kalbasi et al., "Personalized medicine in breast cancer: pharmacogenomics approaches," *Pharmacogenomics and Personalized Medicine*, vol. 12, pp. 59–73, 2019.

[3] A. Prat, E. Pineda, B. Adamo et al., "Clinical implications of the intrinsic molecular subtypes of breast cancer," *The Breast*, vol. 24, no. Suppl 2, pp. S26–S35, 2015.

[4] A. Bettaieb, C. Paul, S. Plenchette, J. Shan, L. Chouchane, and F. Ghiringhelli, "Precision medicine in breast cancer: reality or utopia?" *Journal of Translational Medicine*, vol. 15, no. 1, p. 139, 2017.

[5] U. Krishnamurti and J. F. Silverman, "HER2 in breast cancer," *Advances in Anatomic Pathology*, vol. 21, no. 2, pp. 100–107, 2014.

[6] P. Monteiro Ide, P. Madureira, A. de Vasconscelos, D. H. Pozza, and R. A. de Mello, "Targeting HER family in HER2-positive metastatic breast cancer: potential biomarkers and novel targeted therapies," *Pharmacogenomics*, vol. 16, no. 3, pp. 257–271, 2015.

[7] H. M. Asif, S. Sultana, S. Ahmed, N. Akhtar, and M. Tariq, "HER-2 positive breast cancer-a mini-review," *Asian Pacific Journal of Cancer Prevention*, vol. 17, no. 4, pp. 1609–1615, 2016.

[8] S. Loibl and L. Gianni, "HER2-positive breast cancer," *The Lancet*, vol. 389, no. 10087, pp. 2415–2429, 2017.

[9] N. Iqbal and N. Iqbal, "Human epidermal growth factor receptor 2 (HER2) in cancers: overexpression and therapeutic implications," *Molecular Biology International*, vol. 2014, Article ID 8527489, 9 pages, 2014.

[10] K. B. Pahuja, T. T. Nguyen, B. S. Jaiswal et al., "Actionable activating oncogenic ERBB2/HER2 transmembrane and juxtamembrane domain mutations," *Cancer Cell*, vol. 34, no. 5, pp. 792.e5–806.e5, 2018.

[11] J. D. Sato, T. Kawamoto, A. D. Le, J. Mendelsohn, J. Polikoff, and G. H. Sato, "Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors," *Molecular Biology and Medicine*, vol. 1, pp. 511–529, 1983.

[12] E. Kovacs, J. A. Zorn, Y. Huang et al., "A structural perspective on the regulation of the EGF receptor," *Annual Review of Biochemistry*, vol. 84, pp. 739–764, 2015.

[13] R. Roskoski Jr., "The ErbB/HER family of protein-tyrosine kinases and cancer," *Pharmacological Research*, vol. 79, pp. 34–74, 2014.

[14] C. Desbois-Mouthon, "The HER3/ErbB3 receptor: a promising target in cancer drug therapy," *Gastroentérologie Clinique et Biologique*, vol. 34, no. 4-5, pp. 255–259, 2010.

[15] P. J. Brennan, T. Kumogai, A. Berezov, R. Murali, and M. I. Greene, "HER2/neu: mechanisms of dimerization/oligomerization," *Oncogene*, vol. 19, no. 53, pp. 6093–6101, 2000.

[16] A. Ocaña and A. Pandiella, "An update into the pathophysiological role of HER2 in cancer: therapeutic implications," *Clinical and Translational Oncology*, vol. 9, no. 9, pp. 543-544, 2007.

[17] J. Jeong, J. N. VanHouten, P. Dann et al., "PMCA2 regulates HER2 protein kinase localization and signaling and promotes HER2-mediated breast cancer," *Proceedings of the National Academy of Sciences*, vol. 113, no. 3, pp. E282–E290, 2016.

[18] S. Blasco-Benito, E. Moreno, M. Seijo-Vila et al., "Therapeutic targeting of HER2-CB2R heteromers in HER2-positive breast cancer," *Proceedings of the National Academy of Sciences*, vol. 116, no. 9, pp. 3863–3872, 2019.

[19] C. Gutierrez and R. Schiff, "HER2: biology, detection, and clinical implications," *Archives of Pathology and Laboratory Medicine*, vol. 135, no. 135, pp. 55–62, 2011.

[20] J. Baselga, R. E. Coleman, J. Cortés, and W. Janni, "Advances in the management of HER2-positive early breast cancer," *Critical Reviews in Oncology/Hematology*, vol. 119, pp. 113–122, 2017.

[21] E. Cocco, S. Lopez, A. D. Santin, and M. Scaltriti, "Prevalence and role of HER2 mutations in cancer," *Pharmacology & Therapeutics*, vol. 199, pp. 188–196, 2019.

[22] S. G. Li and L. Li, "Targeted therapy in HER2-positive breast cancer," *Biomedical Reports*, vol. 1, no. 4, pp. 499–505, 2013.

[23] S. Ahmed, A. Sami, and J. Xiang, "HER2-directed therapy: current treatment options for HER2-positive breast cancer," *Breast Cancer*, vol. 22, no. 2, pp. 101–116, 2015.

[24] N. Harbeck, "Advances in targeting HER2-positive breast cancer," *Current Opinion in Obstetrics and Gynecology*, vol. 30, no. 1, pp. 55–59, 2018.

[25] Z. S. Lima, M. Ghadamzadeh, F. T. Arashloo, G. Amjad, M. R. Ebadi, and L. Younesi, "Recent advances of therapeutic targets based on the molecular signature in breast cancer: genetic mutations and implications for current treatment paradigms," *Journal of Hematology & Oncology*, vol. 12, no. 1, p. 38, 2019.

[26] A. Ferrari, A. Vincent-Salomon, X. Pivot et al., "A whole-genome sequence and transcriptome perspective on HER2-positive breast cancers," *Nature Communications*, vol. 7, no. 1, Article ID 12222, 2016.

[27] S. Kurozumi, M. Alsaleem, C. J. Monteiro et al., Targetable ERBB2 mutation status is an independent marker of adverse prognosis in estrogen receptor positive, ERBB2 non-amplified

primary lobular breast carcinoma: a retrospective in silico analysis of public datasets, 2020.

[28] R. Bose, S. M. Kavuri, A. C. Searleman et al., "Activating HER2 mutations in HER2 gene amplification negative breast cancer," *Cancer Discovery*, vol. 3, no. 2, pp. 224–237, 2013.

[29] F. Petrelli, G. Tomasello, S. Barni, V. Lonati, R. Passalacqua, and M. Ghidini, "Clinical and pathological characterization of HER2 mutations in human breast cancer: a systematic review of the literature," *Breast Cancer Research and Treatment*, vol. 166, no. 2, pp. 339–349, 2017.

[30] J. Subramanian, A. Katta, A. Masood, D. R. Vudem, and R. K. Kancha, "Emergence of ERBB2 mutation as a biomarker and an actionable target in solid cancers," *Oncologist*, vol. 24, no. 12, 2019.

[31] C. M. Connell and G. J. Doherty, "Activating HER2 mutations as emerging targets in multiple solid cancers," *ESMO Open*, vol. 2, no. 5, pp. 1–11, 2017.

[32] W.-J. Zuo, Y.-Z. Jiang, Y.-J. Wang et al., "Dual characteristics of novel HER2 kinase domain mutations in response to HER2-targeted therapies in human breast cancer," *Clinical Cancer Research*, vol. 22, no. 19, pp. 4859–4869, 2016.

[33] D. M. Hyman, S. A. Piha-Paul, H. Won et al., "HER kinase inhibition in patients with HER2- and HER3-mutant cancers," *Nature*, vol. 554, no. 7691, pp. 189–194, 2018.

[34] Z. Sun, Y. Shi, Y. Shen, L. Cao, W. Zhang, and X. Guan, "Analysis of different HER-2 mutations in breast cancer progression and drug resistance," *Journal of Cellular and Molecular Medicine*, vol. 19, no. 12, pp. 2691–2701, 2015.

[35] S. Croessmann, L. Formisano, L. N. Kinch et al., "Combined blockade of activating ERBB2 mutations and ER results in synthetic lethality of ER+/HER2 mutant breast cancer," *Clinical Cancer Research*, vol. 25, no. 1, pp. 277–289, 2019.

[36] U. Nayar, O. Cohen, C. Kapstad et al., "Acquired HER2 mutations in ER+ metastatic breast cancer confer resistance to estrogen receptor-directed therapies," *Nature Genetics*, vol. 51, no. 2, pp. 207–216, 2019.

[37] CBIOPORTAL, v3. 1.0, https://www.cBioPortal.org/.

[38] COSMIC, v90, http://cancer.sanger.ac.uk/cosmic.

[39] PubMed database in https://www.ncbi.nlm.nih.gov/pubmed.

[40] R. Mishra, A. B. Hanker, and J. T. Garrett, "Genomic alterations of ERBB receptors in cancer: clinical implications," *Oncotarget*, vol. 8, no. 69, pp. 114371–114392, 2017.

[41] X. Xu, C. De Angelis, K. A. Burke et al., "HER2 reactivation through acquisition of the HER2 L755S mutation as a mechanism of acquired resistance to HER2-targeted therapy in HER2+ breast cancer," *Clinical Cancer Research*, vol. 23, no. 17, pp. 5123–5134, 2017.

[42] G. Cocco, F. Javier Carmona, P. Razavi et al., "Neratinib is effective in breast tumors bearing both amplification and mutation of ERBB2 (HER2)," *Science Signaling*, vol. 11, no. 551, Article ID eaat9773, 2018.

[43] R. K. Kancha, N. von Bubnoff, N. Bartosch, C. Peschel, R. A. Engh, and J. Duyster, "Differential sensitivity of ERBB2 kinase domain mutations towards lapatinib," *PLoS One*, vol. 6, no. 10, Article ID e26760, 2011.

[44] M. Nagano, S. Kohsaka, T. Ueno et al., "High-throughput functional evaluation of variants of unknown significance in ERBB2," *Clinical Cancer Research*, vol. 24, no. 20, pp. 5112–5122, 2018.

[45] Y. Hirotsu, H. Nakagomi, K. Amemiya et al., "Intrinsic HER2 V777L mutation mediates resistance to trastuzumab in a breast cancer patient," *Medical Oncology*, vol. 34, no. 1, 2017.

[46] J. Shih, B. Bashir, K. S. Gustafson et al., "Cancer signature investigation: ERBB2 (HER2)-activating mutation and amplification-positive breast carcinoma mimicking lung primary," *Journal of the National Comprehensive Cancer Network*, vol. 13, no. 8, pp. 947–952, 2015.

[47] C. K. Y. Ng, L. G. Martelotto, A. Gauthier et al., "Intra-tumor genetic heterogeneity and alternative driver genetic alterations in breast cancers with heterogeneous HER2 gene amplification," *Genome Biology*, vol. 16, no. 1, pp. 1–21, 2015.

[48] X. Han, L. Diao, Y. Xu et al., "Association between the HER2 Ile655Val polymorphism and response to trastuzumab in women with operable primary breast cancer," *Annals of Oncology*, vol. 25, no. 6, pp. 1158–1164, 2014.

[49] H. Singla, R. P. Kaur, G. Shafi et al., "Genomic alterations associated with HER2+ breast cancer risk and clinical outcome in response to trastuzumab," *Molecular Biology Reports*, vol. 46, no. 1, pp. 823–831, 2019.

[50] L. Roca, V. Diéras, H. Roché et al., "Correlation of HER2, FCGR2A, and FCGR3A gene polymorphisms with trastuzumab related cardiac toxicity and efficacy in a subgroup of patients from UNICANCER-PACS04 trial," *Breast Cancer Research and Treatment*, vol. 139, no. 3, pp. 789–800, 2013.

[51] A. Madrid-Paredes, M. Cañadas-Garre, A. Sánchez-Pozo et al., "ABCB1 C3435T gene polymorphism as a potential biomarker of clinical outcomes in HER2-positive breast cancer patients," *Pharmacological Research*, vol. 108, pp. 111–118, 2016.

[52] D. Furrer, S. Jacob, A. Michaud, L. Provencher, J. Lemieux, and C. Diorio, "Association of tobacco use, alcohol consumption and HER2 polymorphisms with response to trastuzumab in HER2-positive breast cancer patients," *Clinical Breast Cancer*, vol. 18, no. 4, pp. e687–e694, 2018.

[53] D. J. Serie, J. E. Crook, B. M. Necela et al., "Genome-wide association study of cardiotoxicity in the NCCTG N9831 (Alliance) adjuvant trastuzumab trial," *Pharmacogenetics and Genomics*, vol. 27, no. 10, pp. 378–385, 2017.

[54] Y. Endo, Y. Dong, N. Kondo et al., "HER2 mutation status in Japanese HER2-positive breast cancer patients," *Breast Cancer*, vol. 23, no. 6, pp. 902–907, 2016.

[55] L. Cabel, A. Fuerea, L. Lacroix et al., "Efficacy of histology-agnostic and molecularly-driven HER2 inhibitors for refractory cancers," *Oncotarget*, vol. 9, no. 11, pp. 9741–9750, 2018.

[56] G. Deniziaut, J. C. Tille, F.-C. Bidard et al., "ERBB2 mutations associated with solid variant of high-grade invasive lobular breast carcinomas," *Oncotarget*, vol. 7, no. 45, pp. 73337–73346, 2016.

[57] T. Wang, Y. Xu, S. Sheng et al., "HER2 somatic mutations are associated with poor survival in HER2-negative breast cancers," *Cancer Science*, vol. 108, no. 4, pp. 671–677, 2017.

[58] M. T. Chang, T. S. Bhattarai, A. M. Schram et al., "Accelerating discovery of functional mutant alleles in cancer," *Cancer Discovery*, vol. 8, no. 2, pp. 174–183, 2018.

[59] S. M. Ali, R. K. Alpaugh, S. R. Downing et al., "Response of an ERBB2-mutated inflammatory breast carcinoma to human epidermal growth factor receptor 2-targeted therapy," *Journal of Clinical Oncology*, vol. 32, no. 25, pp. e88–e91, 2014.

[60] M. Christgen, S. Bartels, A. Luft et al., "Activating human epidermal growth factor receptor 2 (HER2) gene mutation in bone metastases from breast cancer," *Virchows Archiv*, vol. 473, no. 5, pp. 577–582, 2018.

[61] D. J. Zabransky, C. L. Yankaskas, R. L. Cochran et al., "HER2 missense mutations have distinct effects on oncogenic signaling and migration," *Proceedings of the National Academy of Sciences*, vol. 112, no. 45, pp. E6205–E6214, 2015.

[62] J. P. Robichaux, Y. Y. Elamin, R. S. K. Vijayan et al., "Pan-cancer landscape and analysis of ERBB2 mutations identifies poziotinib as a clinically active inhibitor and enhancer of T-DM1 activity," *Cancer Cell*, vol. 36, no. 4, pp. 444.e7–457.e7, 2019.

[63] S. Verma, S. Goyal, A. Kumari, A. Singh, S. Jamal, and A. Grover, "Structural investigations on mechanism of lapatinib resistance caused by HER-2 mutants," *PLoS One*, vol. 13, no. 2, Article ID e0190942, 2018.

[64] http://cancerhotspots.org.

[65] S. J. Fleishman, J. Schlessinger, and N. Ben-Tal, "A putative molecular-activation switch in the transmembrane domain of erbB2," *Proceedings of the National Academy of Sciences*, vol. 99, no. 25, pp. 15937–15940, 2002.

[66] F. C. de Almeida, B. K. Banin Hirata, C. B. Ariza et al., "HER2Ile655Val polymorphism is negatively associated with breast cancer susceptibility," *Journal of Clinical Laboratory Analysis*, vol. 32, no. 6, Article ID e22406, 2018.

[67] B. M. Krishna, S. Chaudhary, A. K. Panda, D. R. Mishra, and S. K. Mishra, "Her2 Ile655Val polymorphism and its association with breast cancer risk: an updated meta-analysis of case-control studies," *Scientific Reports*, vol. 8, no. 1, p. 7427, 2018.

[68] Y. Ogoshi, K. Shien, T. Yoshioka et al., "Anti-tumor effect of neratinib against lung cancer cells harboring HER2 oncogene alterations," *Oncology Letters*, vol. 17, no. 3, pp. 2729–2736, 2019.

[69] S. Jasra, M. Opyrchal, L. Norton, and R. Mehta, "A rare case of S310F somatic ERBB2 mutation in a HER2-nonamplified breast cancer," *Clinical Breast Cancer*, vol. 17, no. 1, pp. e37–e41, 2017.

[70] S. M. Kavuri, N. Jain, F. Galimi et al., "HER2 activating mutations are targets for colorectal cancer treatment," *Cancer Discovery*, vol. 5, no. 8, pp. 832–841, 2015.

[71] J. Wang, Y. Wen, G. Ding et al., "Efficacy generated by afatinib in a lung adenocarcinoma patient harboring HER2 S310Y mutation," *Cancer Biology & Therapy*, vol. 19, no. 6, pp. 450–453, 2018.

[72] O. Vornicova, D. Hershkovitz, T. Yablonski-Peretz, O. Ben-Itzhak, Z. Keidar, and G. Bar-Sela, "Treatment of metastatic extramammary paget's disease associated with adnexal adenocarcinoma, with anti-HER2 drugs based on genomic alteration ERBB2 S310F," *The Oncologist*, vol. 19, no. 9, pp. 1006-1007, 2014.

[73] H. Greulich, B. Kaplan, P. Mertins et al., "Functional analysis of receptor tyrosine kinase mutations in lung cancer identifies oncogenic extracellular domain mutations of ERBB2," *Proceedings of the National Academy of Sciences*, vol. 109, no. 36, pp. 14476–14481, 2012.

[74] J. W. Shin, S. Kim, S. Ha et al., "The HER2 S310F mutant can form an active heterodimer with the EGFR, which can Be inhibited by cetuximab but not by trastuzumab as well as pertuzumab," *Biomolecules*, vol. 9, no. 10, p. 629, 2019.

[75] O. L. Griffith, N. C. Spies, M. Anurag et al., "The prognostic effects of somatic mutations in ER-positive breast cancer," *Nature Communications*, vol. 9, no. 1, p. 3476, 2018.

[76] M. de Martino, D. Zhuang, T. Klatte et al., "Impact of ERBB2 mutations on in vitro sensitivity of bladder cancer to lapatinib," *Cancer Biology & Therapy*, vol. 15, no. 9, pp. 1239–1247, 2014.

[77] C. X. Ma, R. Bose, F. Gao et al., "Neratinib efficacy and circulating tumor DNA detection of HER2 mutations in HER2 nonamplified metastatic breast cancer," *Clinical Cancer Research*, vol. 23, no. 19, pp. 5687–5695, 2017.

[78] Z. Yi, F. Ma, Y. Guan et al., "Impact of HER2 mutation status on personalized molecular targeted therapy in advanced breast cancers," *Journal of Clinical Oncology*, vol. 36, no. 15_suppl, p. 1039, 2018.

Research Article

Circulating Tumor DNA Using Tagged Targeted Deep Sequencing to Assess Minimal Residual Disease in Breast Cancer Patients Undergoing Neoadjuvant Chemotherapy

Gabriella Cirmena,[1] Anna Garuti,[1] Marilena De Mariano,[1] Simona Coco,[2] Lorenzo Ferrando,[1] Edoardo Isnaldi,[1] Valentina Barbero,[3] Piero Fregatti,[3,4] Lucia Del Mastro,[1,3] Fabio Ferrando,[1,3] Roberta Gonella,[1,3] Alessandro Garlaschi,[3,4] Daniele Friedman,[3,4] Alberto Ballestrero,[1,3] and Gabriele Zoppoli [1,3]

[1]*Department of Internal Medicine, University of Genoa, 16132 Genoa, Italy*
[2]*Lung Cancer Unit, Ospedale Policlinico San Martino, 16132 Genoa, Italy*
[3]*Ospedale Policlinico San Martino, 16132 Genoa, Italy*
[4]*Department of Surgical Sciences and Integrated Diagnostic DISC, 16132 Genoa, Italy*

Correspondence should be addressed to Gabriele Zoppoli; gabriele.zoppoli@unige.it

In breast cancer patients undergoing neoadjuvant chemotherapy before surgery, there is an unmet need for noninvasive predictive biomarkers of response. The analysis of circulating tumor DNA (ctDNA) in particular has been the object of several reports, but few of them have studied the applicability of tagged targeted deep sequencing (tTDS) to clinical practice and its performance compared with droplet digital PCR (ddPCR). Here, we present the first results from an ongoing study involving a prospectively accrued, monocentric cohort of patients affected by invasive breast cancer, undergoing neoadjuvant chemotherapy followed by surgery with curative intent as per clinical practice. A pretreatment tumor biopsy and plasma samples were collected before and during treatment, after surgery, and every six months henceforth or until relapse, whichever came first. Pretreatment biopsies were sequenced with a 409-gene massive parallel sequencing (MPS) panel, allowing the identification of target mutations and their research in plasma by tTDS and ddPCR as a complementary approach. Using tTDS, we demonstrated the presence of at least one deleterious mutation in all the relapsed cases we studied ($n = 4$), with an average lead time of six months before clinical relapse. The association with ddPCR was suboptimal, and only one relapsed patient could be identified with such method. tTDS shows potential as an early noninvasive method for the detection of MRD in BC patients.

1. Introduction

Breast cancer (BC) is the most common type of cancer in women worldwide, and approximately 30% of patients initially diagnosed with early-stage cancer will eventually develop metastatic disease [1]. Therefore, there is an urgent need for biomarkers to identify minimal residual disease (MRD) and to better assess the risk of relapse during patients' follow-up. Circulating tumors cells (CTCs) were approved by the Federal Drug Agency (FDA) for the prognostic stratification of early breast cancer patients, but the sensitivity of this method is relatively low and the retrieval of CTC is not a trivial task [2, 3]. Recently, the detection of circulating tumor DNA (ctDNA) in the blood has showed promise in providing prognostic and predictive information for the clinical management of breast cancer patients [4–6]. The use of ctDNA has advantages over the use of tissue biopsies due to its availability with minimally invasive procedures, and the opportunity to obtain multiple samples over several time points. Therefore, sensitive

assessment of the presence of ctDNA may represent an ideal biomarker for the purposes outlined above [7]. Efforts to detect ctDNA have intensified in recent years [8–10], and the advent of massively parallel sequencing (MPS) has provided unprecedented opportunities as well as threats in such regard [11–13].

On the one hand, ctDNA tracking over time can constitute the basis of advanced personalized treatment and could be used to monitor the presence of MRD. On the other hand, benchmarking and optimization of new technical platforms for ctDNA detection are mandatory before its introduction into clinical practice. First of all, the control of various parameters, from blood collection to isolation of circulating DNA, has a significant impact on the quality and accuracy of the data. Tumor-specific digital droplet polymerase chain reaction (ddPCR) assays are highly accurate, but require the design and optimization of personalized assays, an expensive, time-consuming, and not always successful step. Furthermore, genetic aberrations of metastatic disease may differ from those found in the primary tumor [14]. Tagged targeted deep sequencing of plasma DNA (tTDS), a novel proprietary method now under active development [15, 16], could provide a better alternative for high-throughput analysis of ctDNA compared with ddPCR and may overcome limitations of initial tumor tissue assessment allowing for the direct identification of several low-frequency ctDNA mutations.

Here, we present the results of tTDS using Oncomine™ Breast v2 cfDNA Assays (Thermo Fisher Scientific™) in BC patients undergoing neoadjuvant chemotherapy (NACT). The aims of the present study were (1) to demonstrate the feasibility of obtaining circulating cell-free DNA (ccfDNA) from plasma samples suitable for NGS by performing appropriate quality controls and (2) to develop an optimized workflow for mutation tracking in serial plasma samples to predict treatment response and/or early relapse in BC patients undergoing NACT followed by surgery with curative intent as per clinical practice.

2. Patients and Methods

2.1. Patients.
Inclusion criteria were: histological diagnosis of invasive breast cancer with indication to NACT as per clinical practice, completion of at least 85% of NACT; availability of enough material from the diagnostic biopsy at diagnosis for NGS assessment; availability of plasma samples at the specified time points, i.e., basal, half treatment completed, before surgery, at 12 weeks, 24 weeks, 1 year after surgery, and every 24 weeks until 24 months of follow-up or upon relapse; willingness to participate; and written informed consent. Exclusion criteria were: death from noncancer-related causes within the first 12 months after surgery; unwillingness or inability to participate; multicentric/bilateral disease; pT1mic/pN0 at surgery, due to the scarcity of data concerning the prognostic value of such therapeutic result; diagnosis of advanced disease within six months of diagnosis; and refusal to participate or consent withdrawal.

BC patients undergoing NACT were recruited between 2014 and 2018. All patients underwent pretreatment clinical and radiological staging before treatment, as per clinical practice. Baseline radiological staging of all patients was obtained by magnetic resonance and whole-body CT, and expressions of Ki-67, estrogen receptor (ER), progesterone receptor (PR), and HER2 status were assessed locally as per SIAPEC/ASCO/CAP criteria. Surgical and histopathological findings such as tumor subtype as well as clinical data of direct relevance for our study and prespecified per protocol were also recorded. After completion of NACT, patients underwent surgery. The histopathological findings at surgery were compared with pretreatment staging. Pathological complete response (pCR) was defined as the complete absence of invasive tumor in the primary site and excised lymph nodes. *In situ* neoplasia (Tis) was not considered as invasive disease.

2.2. Extraction of DNA from Biopsies and PBMCs.
DNA from formalin-fixed paraffin-embedded (FFPE) breast tissue samples was extracted using the Maxwell® RSC DNA FFPE Kit with the Maxwell® RSC Instrument (Promega Corporation, Madison, USA) according to the manufacturer's instructions. Germline DNA from peripheral blood mononuclear cells (PBMCs) was extracted using the Maxwell® 16 Blood DNA Purification Kit according to the manufacturer's instructions.

The concentration and purity of DNA samples were measured using both a NanoDrop™ 2000 spectrophotometer (Thermo Scientific™) and a Qubit™ dsDNA HS Assay Kit (Invitrogen™) designed for use with the Qubit™ 2.0 Fluorometer (Invitrogen™).

2.3. Mutational Analysis by MPS on Biopsy FFPE and PBMCs.
The Comprehensive Cancer Panel™ (CCP, IonAmpliSeq™ Comprehensive Cancer Panel™, Thermo Fisher Scientific™) was used to identify target mutations in exonic regions of 409 cancer-related genes. Four libraries were created using 40 ng from both FFPE DNA from diagnostic biopsies and germline DNA samples as per manufacturer's specification [17] Libraries were quality-checked on an Agilent™ TapeStation. The Thermo Fisher Scientific™ Ion Chef™ system was used for template preparation followed by sequencing on an Ion PGM™ System using Ion 318™ chips, one library per chip for FFPE DNA samples and four germline libraries per chip [18].

2.4. Isolation, Extraction, and Quantification of Circulating Cell-Free DNA (ccfDNA).
Blood samples were collected in BD Vacutainer® EDTA tubes (Becton Dickinson). To separate plasma, whole blood was processed within 2 hours by centrifugation for 10 min at 1600 ×g and collected in a new conical tube. Plasma was then recentrifuged for further 10 min at 1600 ×g. The resulting plasma was stored at −80° until DNA extraction.

As per manufacturer's specifications, 10 nanograms of ccfDNA were used for ddPCR experiments and 20 ng for tTDS experiments. To ensure that such quantities were obtained, we collected three 7.5 ml peripheral blood test

tubes for each patient. We then proceeded to extract ccfDNA from 2 to 5 ml of plasma from each of the two tubes according to individual yield. The third was stored as back-up in case of extraction failure of the need for further experiments. For extraction, we used the QIAamp Mini Elute cfDNA Mini or Midi Kit (Qiagen, Hilden, Germany) on the QIAcube system, according to the manufacturer's instructions. DNA was eluted into 40–50 μl Ultraclean water in DNA Lo Bind tubes and stored at −20°C. Isolated cfDNA was then quantified by Qubit™ dsDNA HS Assay Kit (Invitrogen™) designed for use with the Qubit™ 2.0 Fluorometer (Invitrogen™) and for evaluation of fragment size on High Sensitivity D1000 ScreenTape for use with the TapeStation 2200 (Agilent Technologies, Germany).

2.5. Mutational Analysis by ddPCR on ctDNA.

The gene mutations identified by NGS were validated by droplet digital PCR (ddPCR) on ctDNA samples samples using a QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Reactions were performed in 24 μl volumes using 12 μl ddPCR 2x Supermix (Bio-Rad Laboratories, Inc.), 1 μl of 20x assay mix (9 μm primers, 5 μm TaqMan mutant Probe (Bioclarma S.r.l. Research and Molecular Diagnostics, Torino, Italy), and 2.5–10 μl of ctDNA with a final concentration of 10 ng.

For each sample , 20 μl of PCR reaction and 70 μl of droplet generation oil were dispensed into specific wells of a DG8 cartridge and then loaded in the QX100 droplet (Bio-Rad Laboratories, Inc.). Then, 50 μl of droplets were then transferred into the wells of a 96-well PCR plate, sealed, and loaded into the Mastercycler nexus gradient thermal cycler (Eppendorf, Hanburg, Germany) [19]. After PCR was completed, the plate was loaded into the droplet reader (Bio-Rad Laboratories, Inc.) to acquire the droplets. The data were analysed using the proprietary QuantaSoft software (Bio-Rad Laboratories, Inc.). Each PCR plate included a human reference DNA (Promega corporation, Madison, WI) as wild-type control (WTC), a synthetic DNA specific for each mutation (Bioclarma S.r.L.) as positive control (PC), and a nontemplate control (NTC). Each ctDNA sample was run in duplicate, and the allele fraction was calculated as merged of replicates. For each mutation, the threshold was set manually based on signals of NTC, WTC, and PC. A mutation was only considered to be present if three or more FAM positive droplets were detected [20, 21].

2.6. Mutational Analysis by tTDS on FFPE Biopsy and ctDNA.

We used the next-generation sequencing Oncomine™ Breast v2 cfDNA Assays (Thermo Fisher Scientific™) which is designed, under manufacturer's specifications, to detect somatic mutations in plasma down to a limit of detection (LOD) of 0.1% [22] in genes relevant to solid tumors. In the commercial panel, the following genes are investigated: hotspot genes (i.e., with mutations detectable only in already known regions) are AKT1, EGFR, ERBB2, ERBB3, ESR1, FBXW7, KRAS, PIK3CA, SF3B1, and TP53 (~152 hotspots). Copy number genes (CNVs) included in the kit are CCND1, ERBB2, and FGFR1. TP53 has an 88% in silico coverage of the

exonic regions. The library size was checked using the Agilent High Sensitivity DNA Kit by TapeStation 2200 instrument, and library concentration was evaluated with a Qubit® 2.0 Fluorometer using Qubit™ dsDNA HS Assay Kit. Each barcoded library was diluted to 50 pM concentration for template preparation on an Ion Chef System and sequenced on an Ion 540™ chip on the Ion S5 System to obtain a coverage ≥25,000x, as per manufacturer's specifications.

2.7. Power Considerations, Data Processing, and Statistical Analyses.

Assuming an actionable mutation could be found in plasma before surgery in 70% of NACT patients without pCR and <10% of patients with pCR, with a two-sided $\alpha = 0.05$ and $1 - \beta = 0.90$, further assuming a pCR rate of 30%, we could reject the null hypothesis of finding ctDNA in 50% of patients regardless of pCR by analysing 10 patients undergoing NACT, with a 70/30 ratio (3 with pCR and 7 without). Accounting for the failure of obtaining and/or properly analysing the required samples at all time points of 50%, and a refusal to participate or consent withdrawal of 30%, at least 30 patients needed to be screened to proceed to the required analyses.

For identification and interpretation of genetic variants, we matched somatic DNA from biopsies and germline DNA sequencing results, in order to identify truncal, high allelic frequency somatic, and pathogenic mutations. For target mutation selection, in case of multiple variants fulfilling these requirements, we chose the one with the highest variant allelic frequency (VAF) and the greatest coverage. Moreover, we removed variants in genes with reported mutational sequencing in TCGA (provisional version, last accessed on cBioPortal in 2017) <3%.

We used the Ion Reporter® software for mutational analysis by MPS on biopsy FFPE and PBMCs, workflow version 5.10 AmpliSeq CCP v1.1—Tumor-Normal pair, with functional filters as follows: confident somatic variants included; location in exonic; $0.05 \leq$ allele ratio ≤ 1.0; variant type in SNV, INDEL, MNV, LONGDEL, CNV; UCSC common SNPs = not included.

Oncomine™ panels on liquid biopsy samples and tumor biopsies were analysed first with the Torrent Suite Software version 5.4, using the ctDNA Variant Caller plug-in with parameters optimized for "tagseq_cfDNA and tagseq_ffpe, TS version 5.10" application and later with the Ion Reporter® software, with the following workflows: version 5.10 Oncomine TagSeq Breast v2 Liquid Biopsy workflow 2.1—Single Sample, functional filter Oncomine 5.10, and version 5.10 Workflow Oncomine TagSeq Breast v2 Tumor workflow 2.1—Single Sample following standard user guides.

3. Results

3.1. Demographics.

We collected pretreatment formalin-fixed, paraffin-embedded tumor biopsies as well as plasma samples from 38 early IBC patients consecutively undergoing NACT followed by surgery with curative intent. Of these patients, 29 donated plasma beyond T0 and 25 had an available pretreatment biopsy, 15 patients had all their

samples collected or relapsed at least six months after surgery. Two patients withdrew from the study. Three patients were metastatic at diagnosis. Samples from the first 10 patients who completed at least 24 months of follow-up or at relapse were analysed. The demographics and pathological data characteristics of the study cohort are presented in Table 1.

MPS, ddPCR, and tTDS were performed on DNA extracted from 10 tumor biopsies. MPS was also performed on germline DNA. Circulating tumor DNA was successfully extracted in all collected plasma samples. ctDNA was analysed by digital droplet PCR and tTDS. A diagram describing the study is reported in Figure 1.

3.2. Individualized Driver Mutations by MPS Analysed by ddPCR for Mutation Tracking.
Ten FFPE tissue biopsies and matched germline DNA were successfully extracted. The median yield was 140.0 ng/μl (min. 72.3; max 267.7). All samples were successfully amplified with the Comprehensive Cancer Panel™. The sequencing metrics for FFPE sequencing runs were as follows: median mapped reads 5,506,603 (IQR 4,432,976–5,6080,223), average base coverage median 316x (IQR 245–367x), and uniformity base coverage (meant as the proportion of bases read deeper than 20% of overall coverage) median percent 91.0 (IQR 85.8–92.8). We identified six somatic mutation variants according to our established selection criteria, in three different genes: TP53, PIK3CA, and GATA3, with VAF ranging from 6.4% to 29.7%. Such mutations were transversally validated in the same samples using ddPCR (see Table 2).

The ddPCR assays we validated were then used to track the presence of those mutations in serial plasma samples, since some of the selected mutations were not covered by Oncomine Breast v2 cfDNA Assay [TP53 c.614A>G; TP53 c158G>A; GATA3 c.1223_12124insA]. MPS and ddPCR analyses had a good level of agreement in baseline tumor DNA concerning the assessment of mutational VAF, except for one patient (UPN4). Indeed, mutations in ctDNA matching those selected by MPS were detected in 80% (4 of 5; 95% CI, 37.5% to 96.3%) of baseline plasma samples (see Table 3). In none of the five cases, we could detect the selected mutations after the first three months of chemotherapy or before surgery. In one relapsed case, we observed the disappearance of mutated TP53 in ctDNA after surgery and its detectability (0.11 copies/μl) at 24 weeks (T4), anticipating distant relapse by six months (see Figure 2). Of interest, in two patients who have not relapsed by the time of the present work, we detected the selected mutations by ddPCR in plasma at 24 months, without signs of invasive disease as per clinical practice.

3.3. Driver Mutations Analysed by MPS and tTDS on FFPE Tumor Samples at Diagnosis.
Ten FFPE tissue biopsies previously analysed by MPS were resequenced by tTDS, using the commercial Oncomine TagSeq Breast v2 Liquid Biopsy panel. We were able to generate libraries from 15 ng to 20 ng of FFPE DNA, with an average of 17.6 ng per sample, and successfully amplified for sequencing of all the assessed samples. FFPE samples were sequenced at a median read coverage of 27,764x (IQR 21,390x–38,557x), with uniquely tagged molecules covered at a median of 1,719x (IQR 1,061.5–2,300). The median limit of detection, as assessed by the manufacturer's software, was 0.60 % (IQR 0.64–0.67%). Concerning regions covered by the designs of both MPS and tTDS, these methods showed a very good level of agreement in baseline tumor DNA assessment, with tTDS identifying all mutations selected by MPS (see Table 4). Of note, using tTDS, we correctly identified the copy number gain of ERBB2 in one patient, as expected by IHC results. Additionally, several low VAF mutations were observed by tTDA, due to its higher sensitivity compared with MPS. These however will not be covered in the present work since we did not perform transversal validation of their actual presence.

3.4. Tracking Mutations in ctDNA with tTDS to Identify Minimal Residual Disease (MRD) and Anticipate Relapse.
An average of 12.2 ng (range 4.6–21.7 ng) could be extracted and used for tTDS library preparation starting from 2 to 5 mL plasma samples. To assess the validity of our tTDS analyses, to each run we added 20 ng of Horizon Control Multiplex I cfDNA Reference Standard, which is designed to include known somatic mutations covered by our tTDS panel at fixed concentrations of 5%, 1%, and 0.1%. To quantify libraries generated by Oncomine Breast v2 cfDNA, we followed the same recommendations as for FFPE samples (see Methods section). The sequencing metrics for cfDNA sample runs were as follows: median read coverage 30,240x (IQR 22,274.25–41,120.25x) and median coverage of individual tagged molecules (defined by the manufacturer as median molecular coverage) 2,094.5x (IQR 1,261.5–3,144.25x). The median LOD was 0.31% (IQR 0.19–0.45%) (see Figure 3).

Of the samples from the six patients who did not relapse, we could not detect any mutation by tTDS in four cases at the last available time point. In two of those cases, we detected ctDNA mutations in plasma samples, and one of the two cases had the same mutation (TP53 R248W) in both plasma and in the pretreatment FFPE specimen at very low VAF. Three relapsed patients out of four had detectable plasma mutations at diagnosis by tTDS, and in two of these three cases, the same mutations could be confirmed in tissue pretreatment samples. Patient UPN1, for whom a mutation in TP53 could be identified and tracked by MPS and ddPCR both at diagnosis and before relapse, could not be studied by tTDS due to the lack of coverage of that genomic region in the Oncomine Breast tTDS panel (see Table 5).

In one patient (UPN6), both the pretreatment FFPE biopsy and ctDNA harboured the same PIK3CA H1047R somatic mutation and also confirmed at baseline by ddPCR, but only tTDS could detect the reappearance of that mutation in plasma six months before (see Figure 4).

Table 1: Demographics and pathological data of the studied cohort.

UPN	Age at diagnosis (years)	Baseline	ER	HER2	Ki-67 (%)	Pre surg stg	Surg stg	pCR
1	37	cT2(40)N1	+	−	40	cT2(35)N0	pT3N2a	No
2	63	cT2(40)N0	+	+	10	cT1(1)cN0	ympT1cN1a	No
3	39	cT2(24)N0	+	−	70	cT0N0	ypTisN0	Yes
4	73	cT2(30)N1	+	−	16	cmT1(8)bN0	ypT1bN1a	No
5	44	cmT2(24)N0	+	+	60	cT0N0	ypCR	Yes
6	55	cT2(43)N1	+	−	10	cT1(12)cN0	ypT1aN0	No
7	61	cT2(22)N0	+	−	90	cT0N0	ypCR	Yes
8	54	cT3(60)N0	+	−	10	cT0N0	ypT1cN0	No
9	44	cT3(72)N1	+	−	45	cT1(10)N0	ypT1cN1a	No
10	32	cT3(90)N1	+	−	70	cT1(12)cN0	ypT3N2a	No

UPN = unique patient number. Baseline = clinical stage at diagnosis, before NACT. Pre surg stg = clinical staging before surgery and after NACT. Surg stg = pathological staging at surgery. pCR = pathological complete response.
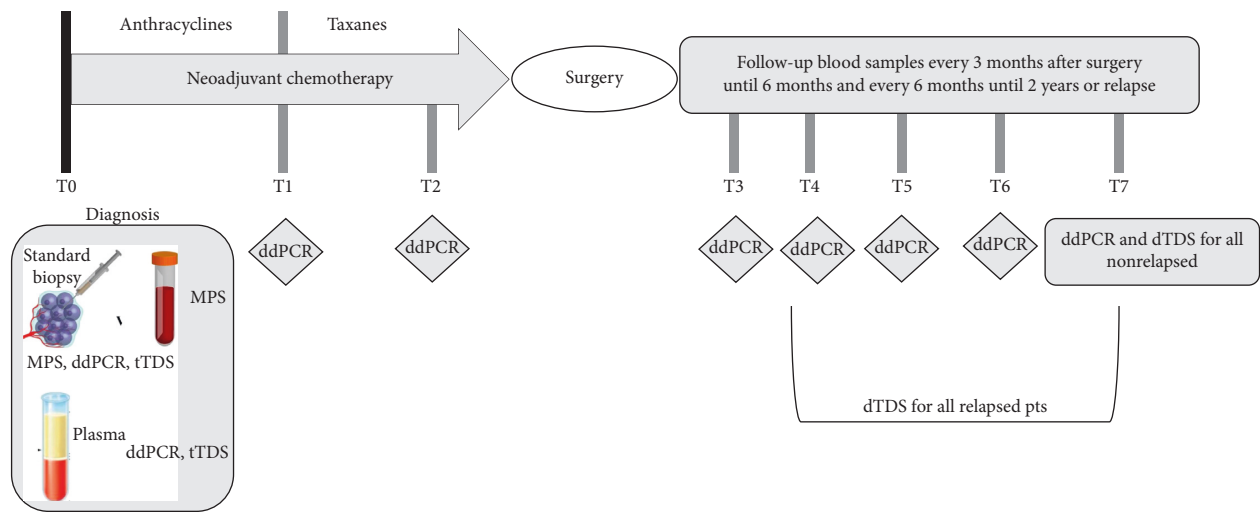
Figure 1: Diagram workflow of the planned sample collection and analyses: at diagnosis, tumor biopsies of patients presenting with invasive breast cancer and indication for NACT were analysed by MPS to identify somatic tumor-specific mutations and then confirmed by ddPCR and tTDS. Mutations in plasma were analysed by ddPCR at baseline, half treatment completed, before surgery, at 12 weeks, 24 weeks, 1 year after surgery, and every 24 weeks until 3 years of follow-up or upon relapse. tTDS was assessed at baseline on biopsies and plasma, as well as at relapse or last follow-up time point.

Table 2: Selected mutations at diagnosis by Comprehensive Cancer Panel™ on FFPE biopsies.

UPN	Genotype	Frequency (%)	Gene	Coding	AA change
1	CAAAT/CAAAC	18.1	TP53	c.614A>G	Tyr205Cys
2	A/G	8.82	PIK3CA	c.1625A>G	Glu542Gly
3			None detected		
4	C/T	21.12	TP53	c.158G>A	Trp53Thr
5			None detected		
6	A/G	29.7	PIK3CA	c.3140A>G	His1047Arg
7			None detected		
8	G/A	6.4	PIK3CA	c.1624G>A	Glu542Lys
9			None detected		
10	C/CA	27.53	GATA3	c.1223_1224insA	Pro409fs

Mutation profile by high-depth targeted MPS in FFPE biopsy of 10 patients: Comprehensive Cancer Panel™ identified somatic genetic alterations in three different genes TP53, PIK3CA, and GATA3 in six patients. According to the stringent selection criteria we established for personalized mutational marker selection, no somatic alterations were found in four patients.

Finally, in one case (UPN8), we could detect mutations at several time points before relapse, but those were not identified in the pretreatment biopsy and varied through time. Interestingly, mutations in KRAS could be found in that case in multiple time points, albeit not in the same genomic position.

TABLE 3: Mutational analysis by ddPCR on FFPE biopsy and on ctDNA.

| Relapsed Patients (UPN) | Target mutation selected by MPS | ddPCR ctDNA sampling | | | |
		Pretreatment (T0)	3 months postsurgery (T3)	Prerelapse	Relapse
1	TP 53 p.Tyr205Cys	Pos	Neg	Pos	Pos
6	PIK3CA p.His1047Arg	Pos	Neg	Neg	Neg
10	GATA3 p.Pro409fs	Pos	Neg	Neg	Neg

Nonrelapsed patients (UPN)	Target mutation selected by MPS	Pretreatment (T0)	3 months postsurgery (T3)	24 months postsurgery (T7)
2	PIK3CA p.Glu542Gly	Pos	Neg	Pos
4	TP 53 p.Trp53Thr	Neg	Neg	Pos

(a)

(b)
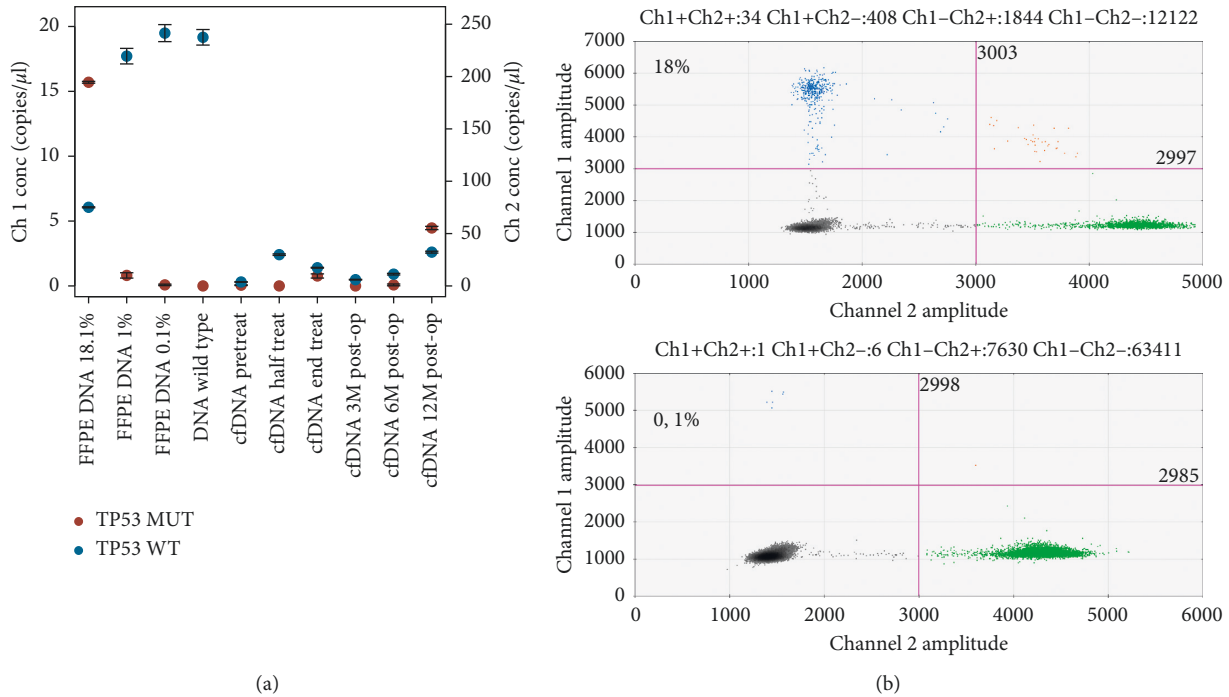
FIGURE 2: ddPCR anticipates clinical relapse in one patient with a TP53 mutation. (a) Assessment of target mutation by ddPCR during the monitoring on ctDNA in a patient (UPN1) relapsed at 12 months. TP53 p. Y205C c.614A>G was present at 6 months (T4) after surgery, anticipating the patient's clinical relapse by six months. (b) ddPCR scatterplot of target mutation (y-axis) vs. wild type (x-axis) on FFPE biopsy at diagnosis (upper plot) and on ctDNA at T4 (lower plot).

TABLE 4: Mutational analysis by MPS and tTDS on FFPE biopsy, comparison between MPS and tTDS on FFPE biopsies samples at diagnosis.

| | | Target mutation selected by MPS (% frequency) | Target mutations selected by tTDS (% VAF) |
		AmpliSeq CCP w1.1—Tumor-Normal pair	Oncomine TagSeq Breast v2 Liquid Biopsy w2.1—Single Sample
Relapsed patients	UPN 1	TP 53 p.Tyr205Cys (18)[#]	TP 53 p.R273H(0.3); ESR1 P.V392I (0.4); TP53 p.V157I (0.4); TP53 p.P82L(0.4)
	UPN 6	PIK3CA p.His1047Arg (29.7)	PIK3CA p.H1047R (23.2); Tp53 p. R282W (1.1)
	UPN 10	GATA3 p.Pro409fs (28.0)[#] TP53c.919 + 1G>C p.?chr 17:7577018(43.0)	TP53c.919 + 1G>C p.?chr 17:7577018 (50.0)
	UPN 8	PIK3CA p.E542K (5.9)	PIK3CA p.E542K (1.5)
Nonrelapsed patients	UPN 2	PIK3CA p.Glu542Gly (9.0)	PIK3CA p.Glu542Gly (8.4)
	UPN 4	TP 53 p.Trp53Thr (21.0)[#]	ERBB3 p. V104M(0.5); TP53 R248W (0.3)
	UPN 5	Nondetected SNV	Nondetected SNV/ERBB2 gain
	UPN 7	TP53c.994 − 1G>C; chr17:7574034 p? (48.2)	TP53 chr17:7574034 p? (48.6); TP53 p. R273H (0.3) CCND1 gain
	UPN 9	Nondetected SNV	FBXW7 p.S582L (0.6)

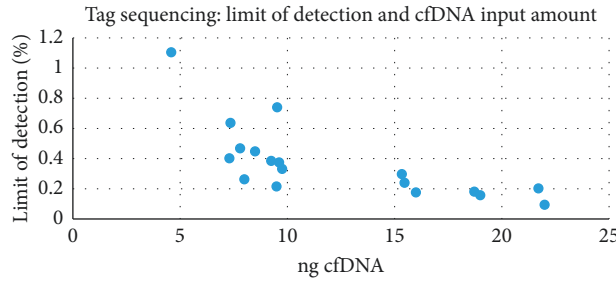[#]Mutation not present in Oncomine Breast cfDNA panel.

FIGURE 3: Scatterplot highlighting the correlation between cfDNA input amount (*x*-axis) and limit of detection (LOD, *y*-axis).

TABLE 5: Mutational analysis by tTDS on ctDNA.

Oncomine Breast liquid Biopsy v2.0 Analysis Ion Reporter: Workflow Oncomine TagSeq Breast v2 Liquid Biopsy w2.1—Single Sample				
A		*FFPE biopsy (% MAF)*	*Prerelapse (% MAF)*	*Relapse (% MAF)*
Relapsed patients	UPN1	TP 53 p.R273H(0,3); ESR1 P.V392I (0,4); TP53 p.V157I (0,3); TP53 p.P82L(0,4)	T4_nondetected SNV	T5_nondetected SNV
	UPN6	PIK3CA p.H1047R (23,2); Tp53 p. R282W (1,1)	T5_ PIK3CA H1047R (0,1); PIK3CA E726K (0,2); Tp53 p. R282W (0,1)	T6_PIK3CA H1047R (0,2)
	UPN 10	TP53 c.919 + 1G>C p.? chr17:7577018 (50)N,P	T5_ CCND1 loss; T7_TP53 c.919 + 1G>C p.? chr17:7577018 (0,2) N,P	T8_not available
	UPN 8	PIK3CA p.E542K (1,5)	T4_KRAS G12D (0,1)	T5_G12V(0,4); T6 _G12V (0,2)
B		*FFPE biopsy (% MAF)*	*Follow-up 24 months (T7) (% MAF)*	
Nonrelapsed patients	UPN2	PIK3CA p.Glu542Gly (8,4)	Nondetected SNV	
	UPN 4	ERBB3 p. V104M(0,5); TP53 R248W (0,3)	TP53 p.R248W (3,9); PIK3CA H1047R (0,6)	
	UPN 3	Nondetected SNV	Nondetected SNV	
	UPN 7	TP53 chr17:7574034 p? (48,6)N,P; TP53 p.R273H(0,3)_CCND1 gain	TP53 p.R273H (1,1); PIK3CA H1047R (0,3); KRAS p.G12A (0,4)	
	UPN 5	Nondetected SNV_ERBB2 gain	Nondetected SNV	
	UPN9	FBXW7 p.S582L(0,6)	Nondetected SNV	

Mutational analysis on ctDNA and FFPE biopsy by tTDS. A: relapsed patients and B: nonrelapsed patients.
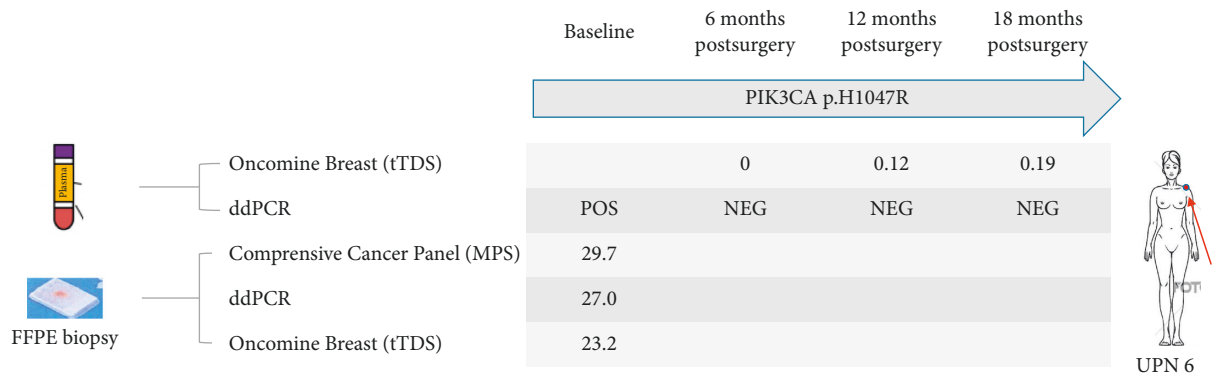

FIGURE 4: Comparison case in point of MPS, MPS, and tTDS for mutation tracking: PIK3CA mutation H1047R could be detected in plasma and in the FFPE pretreatment biopsy by MPS, ddPCR, and tTDS. However, ddPCR allowed the detection of such mutation only at baseline, whereas by tTDS we could track the presence of H1047R six months before relapse.

4. Conclusion

In the present work, we describe the results of a non-interventional, retrospective-prospective case-control study aimed at assessing the presence of mutations in plasma for MRD tracking in patients affected by BC, undergoing NACT for their disease as per clinical practice. To this purpose, we adopted two strategies: (i) targeted MPS of pretreatment FFPE biopsies with a 409-gene panel, followed by stringent selection of an individualized mutation to be assessed as a personalized marker in plasma over time using ddPCR; (ii) evaluation of the presence of mutations in ctDNA using a novel ultrasensitive targeted sequencing approach, namely, tTDS. Our effort allowed us

to identify few mutations answering our criteria in half of our patients' group for ddPCR tracking. With this method, four out of the five patients for whom we designed a ddPCR assay presented with the selected mutation in plasma at baseline. In no case, the personalized tracker mutations were detected after three months of NACT or before surgery, independently from pCR. In only one relapsed case out of four, we could identify with certainty the reappearance of the marker mutation before clinical relapse, with a lead time of six months.

On the other hand, using a commercial tTDS panel—not designed specifically for MRD monitoring—we could detect at least one mutation in three out of four patients who relapsed, usually six months before clinical progression.

We did not have sufficient material left for analysis before surgery, and thus, a comparison with ddPCR cannot be done in this regard. Of interest, by the end of the study, two out of six nonrelapsed patients exhibited the persistence of ctDNA mutations, and these were observed both in plasma and in the pretreatment biopsy using tTDS.

Over the past few years, ctDNA tracking for MRD in BC has been performed with different strategies. Garcia-Murillas et al. [20] described an approach similar, in concept, to what we did. Specifically, however, they assessed snap-frozen pretreatment biopsies using a breast-specific, custom-designed MPS panel. Then, the authors went on designing one or more patient-specific ddPCR assays and used them for personalized monitoring of relapse. Their results were by far more successful than the ones presented in our work, possibly reflecting the context (a prospective clinical trial and the analysis of snap-frozen material), the used panel (a breast-specific one), the choice of multiple ddPCR-personalized probes for monitoring, and a larger cohort of patients. However, that approach was not followed by confirmatory publications, suggesting that, as we experienced, the design of patient-specific ddPCR assays is not an easy task in clinical practice.

Another case-control study [23] assessed the presence of genomic rearrangements in BC surgical specimens by low-pass whole-genome sequencing, and prospectively collected plasma samples were then tested in a time series for the presence of patient-specific translocations assessed by RT-PCR. In the reported work, the authors showed a high degree of sensitivity for the reappearance of genomic aberrations in relapsed patients. Such analysis however came at a high cost in terms of MRD marker personalization and seems of difficult transferability to clinical practice. Of interest however, in that report, the authors identified marker mutations in ctDNA of relapsed patients with a similar lead time to our cases, hinting that the release of appreciable quantities of ctDNA may anticipate clinical relapse by six months in many cases. In that work as in ours, some patients were identified, who presented with ctDNA aberrations without any sign of clinical relapse.

More recently, Cohen et al. [24] presented a large, well-conducted study, in which a tTDS approach using a non-specific cancer panel was adopted together with the search for plasma proteins for the early diagnosis of cancer. The authors reported poor sensitivity with such workflow in BC

and focused their attention to neoplasms that are currently lacking effecting screening strategies, such as pancreatic and ovarian cancer. It has to be noted that the use of a nonbreast tTDS panel may have brought to unsatisfactory results for BC and that the purpose of that work was early noninvasive diagnosis rather than noninvasive monitoring of response and relapse and, as such, it did not have multiple time points available for assessment.

Our study presents several points of weakness: several cases were either lost to follow-up or did not present with all the time points we aimed for. Moreover, we tried to use ddPCR to detect only one potentially trackable mutation, whereas the use of multiple probes may have led to better results in terms of personalized assessment of MRD. The use of a commercial, small tTDS panel not covering key genes frequently mutated in BC such as CDH1 or the whole TP53 exonic region constitutes a strong limitation in assessing the true value of such method in the tested context and serves only as a proof-of-principle analysis of the potential of tTDS in the setting of MRD monitoring. Finally, we do not know whether finding ctDNA mutations in plasma by tTDS, not observed in biopsies of the primary tumor or identified in biopsies but not in plasma, is due to cancer subclonal emergence or to artefacts, and at present, it is difficult to prove or disprove either theory in the absence of a transversal validation method.

Nonetheless, there are several key points in our work that may be of interest for the scientific community, especially for what concerns the pitfalls and caveats of the methods we tested in a clinical practice-like setting. First, the quantity of ccfDNA in nonmetastatic patients was consistently very limited. Retrieving the expected quantity of DNA for both our assays (10 ng is recommended by Bio-Rad for ddPCR and 20 ng by Thermo Fisher for tTDS) was not feasible from a single 7.5 EDTA peripheral blood test tube in most cases. With the recent marketing of larger, ctDNA-optimized tubes, a higher yield is now realistic. Care must still be taken because there are physical limitations—no matter the ctDNA detection method used—to how many mutated molecules we may expect to find in plasma in early BC. Given the weight of a human genome (about 3.6 pg), with a method that may theoretically detect one mutated molecule in 1,000—i.e., a LOD of 0.1%—we will still need at least triple the amount of the DNA from 1,000 nuclei to stand a good statistical chance to observe one mutated DNA fragment in a thousand. We are still not aware of how many molecules may circulate in BC MRD cases, and the fact that, at best, we identified mutations in our patients' ctDNA using either ddPCR or tTDS, with a lead time of six months over clinical relapse suggests that a metastasis just below the detectable size of CT scans—around 0.5 cm—may be the smallest ctDNA-releasing lesion we could identify.

Our study has the strength of comparing head-to-head, albeit in a small case set, the two most promising methods, which are currently available for ctDNA detection. In our experience, MPS followed by the design of ddPCR-personalized assays proved to be more cumbersome than tTDS and did not lead us to more satisfying results than the latter method. Indeed, it is hardly realistic at present to imagine a

clinical practice workflow for early BC patients in which MPS is followed by ddPCR probe design, validation, and use over short periods of time such as monitoring the response to NACT.

This pipeline may be used for monitoring of MRD, but is limited by the identification of one (or more) good candidate target mutation and the successful design of at least one ddPCR probe.

On the other hand, tTDS is characterized by its capability to interrogate hundreds of hotspots simultaneously without the need for optimization in each case. Provided the right conditions are met, especially the presence of a sufficient DNA yield from plasma, tTDS seems of more immediate transferability to clinical practice. With the recent possibility of designing custom tTDS panels, which include the most frequently mutated genes in BC, such as TP53, CDH1, GATA3, and PIK3CA hotspots, the use of this method may lead to an effective way to monitor the presence of MRD in a significant proportion of early BC patients.

In conclusion, our work showed that, in principle, tTDS is a promising technique for the detection of MRD in BC. Further studies should assess its use after target design optimization and by increasing the quantity of plasma to be used for ctDNA detection. Ultimately, the goal of applying tTDS in early BC is, however, to demonstrate not only its clinical validity, but rather its medical utility. This latter task may lead to effective strategies aimed at altering the course of relapsed disease when detected earlier than clinical progression, and studies directed to this purpose are strongly needed.

Abbreviations

BC: Breast cancer
MRD: Minimal residual disease
ctDNA: Circulating tumor DNA
ccfDNA: Circulating cell-free DNA
MPS: Massive parallel sequencing
NACT: Neoadjuvant chemotherapy
tTDS: Tagged targeted deep sequencing
ddPCR: Digital droplet polymerase chain reaction.

Data Availability

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

Conflicts of Interest

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

Authors' Contributions

Alberto Ballestrero and Gabriele Zoppoli equally contributed to this study.

Acknowledgments

References

[1] R. Siegel, C. Desantis, J. Ma, Z. Zou, and A. Jemal, "Colorectal cancer statistics, 2014," *CA: A Cancer Journal for Clinicians*, vol. 64, no. 1, pp. 9–29, 2014.

[2] B. Rack, C. Schindlbeck, J. Juckstock et al., "Circulating tumor cells predict survival in early average-to-high risk breast cancer patients," *Journal of the National Cancer Institute*, vol. 106, no. 5, 2014.

[3] W. Onstenk, J. W. Gratama, J. A. Foekens, S. Sleijfer et al., "Towards a personalized breast cancer treatment approach guided by circulating tumor cell (CTC) characteristics," *Cancer Treatment Reviews*, vol. 39, no. 7, pp. 691–700, 2013.

[4] N. Umetani, A. E. Giuliano, S. H. Hiramatsu et al., "Prediction of breast tumor progression by integrity of free circulating DNA in serum," *Journal of Clinical Oncology*, vol. 24, no. 26, pp. 4270–4276, 2006.

[5] H. Schwarzenbach, "Circulating nucleic acids as biomarkers in breast cancer," *Breast Cancer Research*, vol. 15, no. 5, p. 211, 2013.

[6] A. Ballestrero, A. Garuti, M. Bertolotto et al., "Effect of different cytokines on mammaglobin and maspin gene expression in normal leukocytes: possible relevance to the assays for the detection of micrometastatic breast cancer," *British Journal of Cancer*, vol. 92, no. 10, pp. 1948–1952, 2005.

[7] D. Madhavan, M. Wallwiener, K. Bents et al., "Plasma DNA integrity as a biomarker for primary and metastatic breast cancer and potential marker for early diagnosis," *Breast Cancer Research and Treatment*, vol. 146, no. 1, pp. 163–174, 2014.

[8] K. C. A. Chan, P. Jiang, C. W. M. Chan et al., "Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing," *Proceedings of the National Academy of Sciences*, vol. 110, no. 47, pp. 18761–18768, 2013.

[9] M. W. Snyder, M. Kircher, A. J. Hill, R. M. Daza, and J. Shendure, "Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin," *Cell*, vol. 164, no. 1-2, pp. 57–68, 2016.

[10] K. Sun, P. Jiang, S. H. Cheng et al., "Orientation-aware plasma cell-free DNA fragmentation analysis in open chromatin regions informs tissue of origin," *Genome Research*, vol. 29, no. 3, pp. 418–427, 2019.

[11] K. A. Chan, P. Jiang, Y. W. Zheng et al., "Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing," *Clinical Chemistry*, vol. 59, no. 1, pp. 211–224, 2013.

[12] K. Sun, P. Jiang, K. C. A. Chan et al., "Plasma DNA tissue mapping by genome-wide methylation sequencing for non-invasive prenatal, cancer, and transplantation assessments," *Proceedings of the National Academy of Sciences*, vol. 112, no. 40, pp. E5503–E5512, 2015.

[13] P. Ulz, G. G. Thallinger, M. Auer et al., "Inferring expressed genes by whole-genome sequencing of plasma DNA," *Nature Genetics*, vol. 48, no. 10, pp. 1273–1278, 2016.

[14] D. Brown, D. Smeets, B. Szekely et al., "Phylogenetic analysis of metastatic progression in breast cancer using somatic mutations and copy number aberrations," *Nature Communications*, vol. 8, no. 1, p. 14944, 2017.

[15] C. Vollbrecht, A. Lehmann, D. Lenze, and M. Hummel, "Validation and comparison of two NGS assays for the detection of EGFR T790M resistance mutation in liquid biopsies of NSCLCpatients," *Oncotarget*, vol. 9, no. 26, pp. 18529–18539, 2018.

[16] Y. Kim, S. Shin, and K. A. Lee, "A comparative study for detection of EGFR mutations in plasma cell-free DNA in Korean clinical diagnostic Laboratories," *BioMed Research International*, vol. 2018, Article ID 7392419, 11 pages, 2018.

[17] E. Isnaldi, A. Garuti, G. Cirmena et al., "Clinico-pathological associations and concomitant mutations of the RAS/RAF pathway in metastatic colorectal cancer," *Journal of Translational Medicine*, vol. 17, no. 1, p. 137, 2019.

[18] C. W. Bennett, G. Berchem, Y. J. Kim J, and V. El-Khoury, "Cell-free DNA and next-generation sequencing in the service of personalized medicine for lung cancer," *Oncotarget*, vol. 7, no. 43, pp. 71013–71035, 2016.

[19] B. Zhang, C.-W. Xu, Y. Shao et al., "Comparison of droplet digital PCR and conventional quantitative PCR for measuring EGFR gene mutation," *Experimental and Therapeutic Medicine*, vol. 9, no. 4, pp. 1383–1388, 2015.

[20] I. Garcia-Murillas, G. Schiavon, B. Weigelt et al., "Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer," *Science Translational Medicine*, vol. 7, no. 302, Article ID 302ra133, 2015.

[21] M. Dono, G. De Luca, S. Lastraioli et al., "Tag-based next generation sequencing: a feasible and reliable assay for EGFR T790M mutation detection in circulating tumor DNA of non small cell lung cancer patients," *Molecular Medicine*, vol. 25, no. 1, pp. 1–15, 2019.

[22] Thermo Fisher Scientific Inc, *Oncomine Breast cfDNA Assay—User Guide MAN 0014688*, Thermo Fisher Scientific, Waltham, MA, USA, 2016.

[23] E. Olsson, C. Winter, A. George et al., "Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease," *EMBO Molecular Medicine*, vol. 7, no. 8, pp. 1034–1047, 2015.

[24] J. D. Cohen, L. Li, Y. Wang et al., "Detection and localization of surgically resectable cancers with a multi-analyte blood test," *Science*, vol. 359, no. 6378, pp. 926–930, 2018.

Research Article

Ki-67 Expression is a Significant Prognostic Factor Only When Progesterone Receptor Expression is Low in Estrogen Receptor-Positive and HER2-Negative Early Breast Cancer

Young-Joon Kang [iD],[1] **Han-Byoel Lee,**[2,3] **Yun Gyoung Kim,**[4] **JaiHong Han,**[5] **Yumi Kim,**[2,3] **Tae-Kyung Yoo,**[6] **Eun-Shin Lee,**[2,3] **Hyeong-Gon Moon,**[2,3] **Dong-Young Noh,**[2,3] **and Wonshik Han** [iD][2,3]

[1]*Department of Surgery, Incheon St. Mary's Hospital, The Catholic University of Korea, College of Medicine, Incheon, Republic of Korea*
[2]*Department of Surgery, Seoul National University College of Medicine, Seoul, Republic of Korea*
[3]*Department of Surgery and Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea*
[4]*Department of Surgery, Bundang Jesaeng General Hospital, Seongnam, Republic of Korea*
[5]*Department of Surgery, National Cancer Center, Goyang, Republic of Korea*
[6]*Department of Surgery, Seoul St. Mary's Hospital, The Catholic University of Korea, College of Medicine, Seoul, Republic of Korea*

Correspondence should be addressed to Wonshik Han; hanw@snu.ac.kr

Objective. While the value of Ki-67 has been recognized in breast cancer, controversy also exists. The goal of this study is to show the prognostic value of Ki-67 according to progesterone receptor (PgR) expression in patients who have estrogen receptor- (ER-) positive, human epidermal growth factor receptor 2- (HER2-) negative early breast cancer. *Methods.* The records of nonmetastatic invasive breast cancer patients who underwent surgery at a single institution between 2009 and 2012 were reviewed. Primary end point was recurrence-free survival (RFS), and secondary end point was overall survival (OS). Ki-67 and PgR were assessed with immunohistochemistry for the tumor after surgery. *Results.* A total of 1848 patients were enrolled in this study. 223 (12%) patients had high (≥10%) Ki-67, and 1625 (88%) had low Ki-67 expression. Significantly worse RFS and OS were observed in the high vs. low Ki-67 expression only when the PgR was low (<20%) ($p < 0.001$ and 0.005, respectively, for RFS and OS). There was no significant difference in RFS and OS according to Ki-67 when the PgR was high ($p = 0.120$ and 0.076). RFS of four groups according to high/low Ki-67 and PgR expression was compared. The low PgR and high Ki-67 expression group showed worst outcome among them ($p < 0.001$). In a multivariate analysis, high Ki-67 was an independent prognostic factor when the PgR was low (HR 3.05; 95% CI 1.50–6.19; $p = 0.002$). *Conclusions.* Ki-67 had a value as a prognostic factor only under low PgR expression level in early breast cancer. PgR should be considered in evaluating the prognosis of breast cancer patients using Ki-67.

1. Introduction

The prognosis of breast cancer patients is highly variable and depends on several characteristics. Breast cancers represent a heterogeneous group of tumors with histopathological, immunohistochemical (IHC), and genetic differences [1–4]. Clinicohistopathological characteristics have long been used

to estimate prognosis and decide on treatment plans. Surrogate approaches that use widely available IHC tests for the estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), and Ki-67 have since been developed [5, 6]. Recently, genomic information has been integrated into the clinic for predicting breast cancer prognosis and deciding on systemic treatment [7, 8].

However, due to high costs and technical issues, genetic tests may not be available in much of the world. Roles of biomarkers are still important for determining if the patient would benefit from a particular treatment.

Increased proliferation is a hallmark of malignant tumors and is an essential parameter for the prediction of therapy response [7]. Ki-67 is a representative proliferative index. Many studies have used Ki-67 as a presumptive independent predictive marker for treatment with prognostic value for the clinical outcome as well as disease-free and overall survival [9–17]. However, there is also a controversy about the use of Ki-67, with some studies suggesting that Ki-67 lacks prognostic value [18, 19].

The Saint Gallen Consensus recognized a distinction between "luminal A-like" and "luminal B-like" tumor and supported the value of Ki-67 for the robust prognostic information it conveys [20]. The development of multigene analysis has enabled more refined definitions of breast cancer subtypes. Patients with IHC-based luminal A tumors in the low PgR group had significantly poorer disease-free survival than those in the high PgR group [21]. A previous study reported that 51.3% of IHC luminal A tumors with PgR expression ≤20% fell within the intrinsic luminal B classification, and that only 30.9% of IHC luminal B tumors with PgR expression >20% were actually intrinsic luminal B tumors [21]. Thus, the PgR-based IHC classification of luminal subtypes used clinically is somewhat inaccurate, and combining this classification with Ki-67 expression might improve diagnostic accuracy.

The goal of this study was to clarify the independent prognostic value of determining Ki-67 expression. To this end, we investigated the relationship between Ki-67 and PgR expression levels in clinical practice and correlated the expression of these markers with clinicopathologic variables.

2. Materials and Methods

2.1. Study Population. The records of 1848 patients with pathologically confirmed invasive breast cancer who underwent surgery at the Department of Surgery, Seoul National University Hospital (SNUH; Seoul, South Korea), between July 2009 and December 2012 were retrospectively collected. Patients with ER-positive and HER2-negative breast cancer were included, irrespective of PgR status. Patients diagnosed with in-situ carcinoma or distant metastasis at initial diagnosis or who previously had surgery for breast cancer were excluded, as were those for whom data on PgR or Ki-67 were unavailable. We did not exclude patients who underwent neoadjuvant systemic therapy. IHC analysis was performed from the tissue using core biopsy in a diagnosis. If information from initial tissues was insufficient, we used permanent sections in the patients who were not received neoadjuvant systemic treatment. Biopsy tissue was obtained from patients who underwent neoadjuvant systemic therapy prior to treatment. The study population comprised patients with a diagnosis of stage I to IIIC according to the AJCC (American Joint Committee on Cancer, 8th edition) pathologic staging system. Recurrence was divided into locoregional and distant. Contralateral

recurrence was not included among recurrent categories in this study. The primary end point was recurrence-free survival (RFS) in relation to Ki-67 and PgR expression status. The follow-up period corresponded to the interval from surgery to the last date of a hospital visit, regardless of the visited department. The secondary end point was overall survival (OS). For follow-ups, electronic medical records of patients were reviewed up to November 2015; deaths were recorded based on reports as of December 2013. RFS was classified into four groups based on correlations with Ki-67 and PgR expression.

2.2. IHC Procedure. ER, PgR, HER2, and Ki-67 expression were determined by IHC in formalin-fixed, paraffin-embedded tissue blocks. Expression levels of hormone receptors, HER2, and Ki-67 were assessed using the avidin-biotin complex technique [22]. Tissues were cut into 4 μm-thick sections, deparaffinized with xylene, rehydrated with a graded ethanol series, and immersed in Tris-buffered saline. Representative sections were immunostained, and more than 10 high-power fields of view were randomly selected and examined under an optical microscope. After antigen retrieval, the sections were incubated with primary antibodies against ER (1DO5; Dako, Denmark; 1 : 50), PgR (PgR636; Dako; 1 : 50), HER2 (CB11; Novocastra Laboratories, upon-Tyne, UK; 1 : 200), and Ki-67 (MIB-1; Dako; 1 : 800) at the indicated dilutions. Sections were then incubated with the biotinylated anti-mouse secondary antibody and stained using streptavidin horseradish peroxidase (Zymed Laboratories, San Francisco, CA, USA). The sections were counterstained with Mayer's hematoxylin, dehydrated, cleared, and then mounted for examination. IHC samples were analyzed by one experienced pathologist at SNUH. The cutoff value used to define low versus high Ki-67 expression was the presence of Ki-67 immunoreactivity in more than 10% of stained nuclei in tumor tissues. The 10% cutoff for Ki-67 was found to have the best predictive value for prognosis at SNUH [23]. Patients were divided into tumors with low (<20%) and high (≥20%) PgR expression. The PgR cutoff of 20% is based on the 2013 Saint Gallen International Breast Cancer Conference [20].

2.3. Statistical Analysis. Patients were divided into low and high Ki-67 groups and low and high PgR groups. Clinicopathologic characteristics were assessed using all pairwise comparisons of groups. Categorical variables were compared using chi-square or Fisher's exact test. Student's t-test was used for comparing continuous variables between two groups. RFS was defined as the interval from the date of operation to the date of the first observation of a recurrence or the last follow-up date without evidence of recurrence. OS was defined as the interval from the date of operation to the date of death or last follow-up. Survival rates were estimated using the Kaplan–Meier method, and differences between two groups were compared using the log-rank test. In univariate and multivariate analyses of survival rates, Cox proportional hazard regression was used with adjustment for various factors. Cox regression analyses were used to

calculate hazard ratios (HRs) and 95% confidence intervals (CIs). Values were two-sided, and statistical significance was defined as a p value <0.05. All statistical analyses were performed using SPSS software, version 21 for Windows (IBM Corp., Chicago, IL, USA).

3. Results

3.1. Clinicopathological Characteristics of Patients. A total of 1,848 patients were enrolled in this study. Clinicopathologic characteristics of analyzed patients were accessed by comparing low and high PgR expression subsets and low and high Ki-67 subsets. The mean age was 49–52 years in each subset. Larger cancers (>2 cm) were more commonly associated with high Ki-67 ($p < 0.001$) and low PgR ($p < 0.001$). Histologic grade (HG) dichotomized samples into low and high subsets, with grade 3 are being classified as high and grade 1 + 2 is classified as low. Node-positive and high-HG samples were also identified in high Ki-67 and low PgR subsets. Significantly, more cases with low PgR underwent neoadjuvant chemotherapy, regardless of Ki-67 status. In addition, more patients with high Ki-67 underwent adjuvant chemotherapy, regardless of PgR status. We also considered tumor characteristics according to the operation method. A total of 719 patients underwent mastectomy, with a larger number of these patients having low PgR expression than high PgR expression. However, there was no significant association between Ki-67 subsets and the frequency of mastectomy. Only PgR subsets were significantly different among axilla surgery types. Recurrence, whether local or distant, was observed in 52 patients. Recurrence was local in 10 cases and distant in 42 cases. Mortality findings are based on public data from the Ministry of the Interior; as of 2013 (last year for which mortality data were examined), 11 patients had died (Table 1).

RFS was significantly better for patients in the low Ki-67 expression group than for those in the high Ki-67 group ($p < 0.001$; Figure 1(a)). RFS was also significantly better for the high PgR expression group than the low PgR expression group ($p = 0.022$; Figure 1(b)).

Interestingly, a subset analysis showed that RFS based on Ki-67 expression status was significantly different in the low PgR subset but not in the high PgR subset. Specifically, RFS of patients with high Ki-67 expression and low PgR expression (<20%) was worse than that in the group with low Ki-67 expression and low PgR expression ($p < 0.001$; Figure 2(a)). On the contrary, among patients in the high PgR expression (≥20%) group, there was no significant difference between high and low Ki-67 groups ($p = 0.120$; Figure 2(b)).

RFS was further analyzed by correlating it with subsets divided into four groups based on Ki-67 and PgR expression. Patients with low PgR and high Ki-67 expression showed the poorest outcome compared with the other three groups ($p < 0.001$; Figure 3).

Multivariate Cox regression models showed that Ki-67 was not significantly associated with high PgR expression status, after adjusting for factors including Ki-67 expression group, age, tumor size, nodal status, and HG (HR 2.03; 95% CI 0.61–6.72; $p = 0.247$; Table 2). For patients in the low PgR subset, Ki-67 was markedly associated with RFS (HR 3.05; 95% CI 1.50–6.19; $p = 0.002$; Table 2). Tumor size was statistically significant with RFS in both PgR status.

An analysis of the secondary end point, OS, similarly showed superior survival in the low Ki-67 subset with low PgR expression ($p = 0.005$; Figure 4(a)) and no significant difference in OS between Ki-67 subsets in the high PgR group ($p = 0.076$; Figure 4(b)).

Mean disease-free survival times were 70 months in low PgR/high Ki-67 subset and 75 months in high PgR/low Ki-67 subset. Mean overall survival times were 74 months and 76 months, respectively, for low PgR/high Ki-67 and high PgR/low Ki-67 subset.

4. Discussion

Here, we evaluated the value of Ki-67 as an independent prognostic factor for recurrence and survival in non-metastatic breast cancer patients with ER-positive and HER2-negative tumors. Consistent with previous reports, our study showed that Ki-67 expression exhibited significant prognostic value, but we further demonstrated that Ki-67 is not always an independent prognostic factor. Specifically, Ki-67 had value as a prognostic factor only in the low PgR expression group. In our study, a comparison of RFS among the four expression subgroups revealed the poorest prognosis in the low PgR high Ki-67 subgroup. And the subset was 120 out of 1848 patients. Therefore, active treatment may be considered in about 6% of patients. In terms of predicting prognosis, our findings suggest that combining the Ki-67 expression level with the PgR expression level improves predictive value. Allison et al. reported a strong correlation between high Oncotype DX Recurrence Scores with grade 3 and low-to-absent PR expression and Ki-67 > 10% [24]. In addition, Thakur et al. demonstrated that high Ki-67 status was significantly correlated with the higher Oncotype DX risk-of-recurrence group (low versus high, $p < 0.001$) [25]. If a genomic analysis is not available, the patient of low PgR and high Ki-67 expression in active treatment can be considered in the ER-positive and HER2-negative breast cancer. However, further work is needed, including independent validation and possibly a prospective study, before these findings can be taken towards clinical translation.

Our paper raises several additional issues. Although Ki-67 has been studied as a prognostic marker in breast cancer for more than two decades, there are controversies surrounding the methods used for determining its expression and the overall analytical validity of published results. Analytical validity refers to the ability of a test to produce reproducible and accurate results. For a marker to have prognostic and predictive value, an evidence-based "optimal" cutoff point is essential. Thus, one reason for controversy surrounding the use of Ki-67 as a marker is the absence of a universally accepted standard cutoff value, which has resulted in the use of different specific threshold values by different laboratories [26]. Our institution

Table 1: Clinical and histopathologic characteristics.

Characteristics	Study population ($n = 1848$)					
	PgR low ($n = 895$)	PgR high ($n = 953$)	p value	Ki-67 low ($n = 1625$)	Ki-67 high ($n = 223$)	p value
Age, mean (range)	52.2 (20–81)	49.2 (22–85)	<0.001	50.9 (22–82)	48.6 (20–85)	<0.001
Menopausal status			<0.001			0.191
Premenopausal	413 (46.2)	604 (63.4)		884 (54.4)	133 (59.6)	
Postmenopausal	463 (51.7)	324 (34.0)		704 (43.3)	83 (37.2)	
Unknown	19 (2.1)	25 (2.6)		37 (2.3)	7 (3.1)	
Histologic type			0.147			0.183
IDCa	830 (92.7)	860 (90.2)		1479 (91.0)	211 (94.6)	
ILCa	30 (3.4)	46 (4.8)		71 (4.4)	5 (2.2)	
Others	35 (3.9)	47 (4.9)		75 (4.6)	7 (3.1)	
Tumor size (cm)			<0.001			<0.001
≤2	452 (50.5)	564 (59.2)		919 (56.6)	97 (43.5)	
>2	443 (49.5)	389 (40.8)		706 (43.4)	126 (56.5)	
LN metastasis			<0.001			0.007
Negative	541 (60.4)	693 (72.7)		1103 (67.9)	131 (58.7)	
Positive	354 (39.6)	260 (27.3)		522 (32.1)	92 (41.3)	
Histologic grade			<0.001			<0.001
Low (I, II)	565 (66.6)	727 (76.3)		1243 (76.5)	80 (35.9)	
High (III)	299 (33.4)	226 (23.7)		382 (23.5)	143 (64.1)	
Breast operation			0.001			0.443
Partial mastectomy	511 (57.1)	618 (64.8)		998 (61.4)	131 (58.7)	
Total mastectomy	384 (42.9)	335 (35.2)		627 (38.6)	92 (41.3)	
Axilla operation			<0.001			0.290
Sentinel node biopsy	491 (54.9)	643 (67.5)		1005 (61.8)	129 (57.8)	
Axilla node dissection	404 (45.1)	303 (31.8)		613 (37.7)	94 (42.2)	
Not done	0 (0.0)	7 (0.7)		7 (0.4)	0 (0.0)	
Recurrence			0.043			<0.001
No	861 (96.2)	935 (98.1)		1589 (97.8)	207 (92.8)	
Locoregional	6 (0.7)	4 (0.4)		7 (0.4)	3 (1.3)	
Distant	28 (3.1)	14 (1.5)		29 (1.8)	13 (5.8)	
Death			0.022			0.009
No	885 (98.9)	951 (99.8)		1618 (99.6)	218 (97.8)	
Yes	9 (1.0)	2 (0.2)		6 (0.4)	5 (2.2)	
Unknown	1 (0.1)	0 (0.0)		1 (0.1)	0 (0.0)	

PgR: progesterone receptor, IDCa: invasive ductal carcinoma, ILCa: invasive lobular carcinoma, and LN: lymph node. Patient outcomes according to Ki-67 and PgR expression.
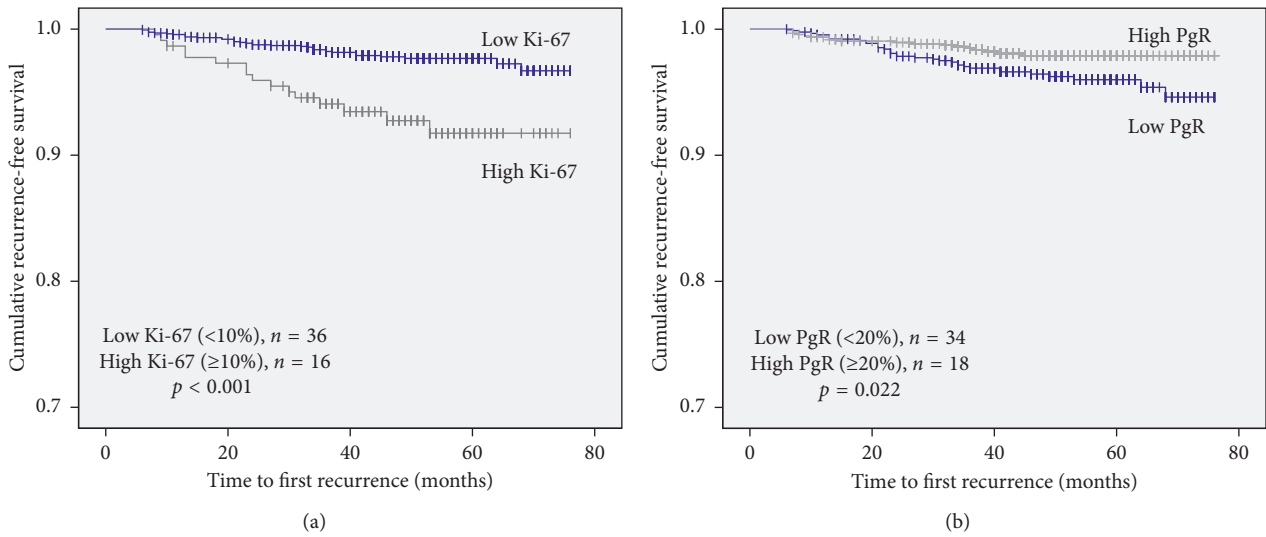
(a)

(b)

FIGURE 1: Recurrence-free survival (RFS) according to the Ki-67 index and progesterone receptor expression status. (a) Overall patients ($n = 1848$); RFS according to Ki-67. (b) Overall patients ($n = 1848$); RFS according to progesterone receptor expression.
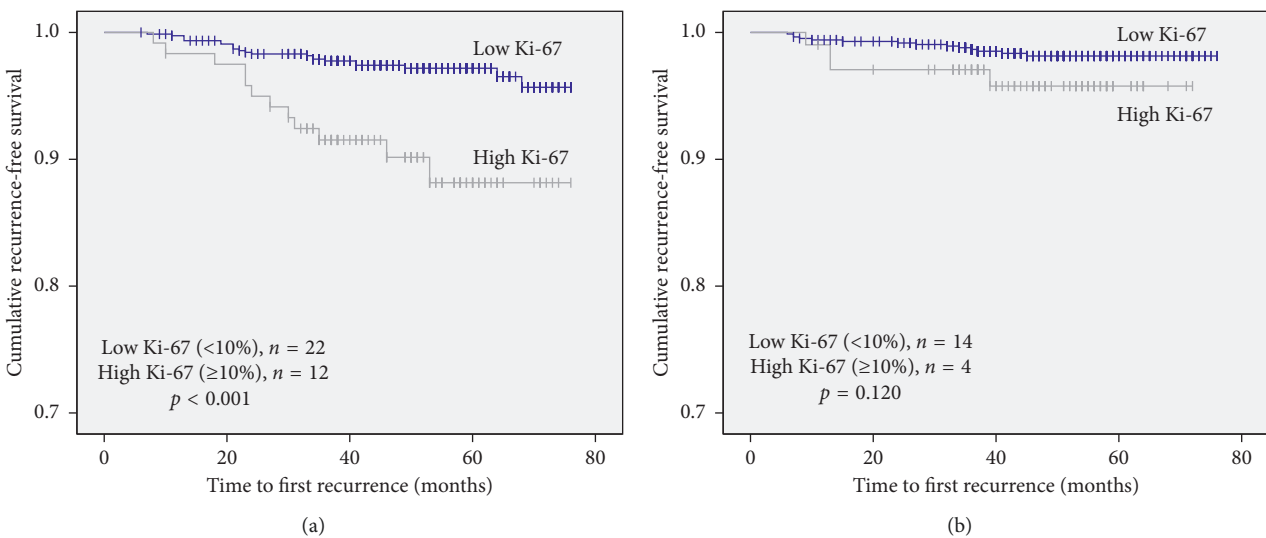

(a)

(b)

FIGURE 2: Recurrence-free survival (RFS) of patients in high and low Ki-67 breast cancer according to progesterone receptor expression status. (a) RFS according to Ki-67 in the low progesterone receptor expression subset (PgR < 20%). (b) RFS according to Ki-67 in the high progesterone receptor expression subset (PgR ≥ 20%).

previously demonstrated that a 10% cutoff value provides the best prognosis-prediction results [23]. This value is different from the cutoff value presented in the Saint Gallen Consensus, which in 2011 defined "low proliferation" tumors as those with a Ki-67 index <14% [5], a cutoff established by comparison with the PAM50 intrinsic multigene molecular test for classification of luminal cancer [27]. During the 2013 Saint Gallen Conference, a majority of panelists voted to raise the threshold indicative of high Ki-67 status to ≥20%. A final definition of a single cutpoint by the Saint Gallen Consensus has remained elusive, owing to the continuous distribution of Ki-67 and analytical and preanalytical barriers to standardized assessment [7]. The cutoff used by our institute is appropriate for our research, but

discussions on standardizing Ki-67 assessments to further reduce interobserver variability will continue. It needs to be analyzed to see whether the results reported in terms of the prognostic value of Ki-67 would be recapitulated if 14% or 20% were used instead of 10%. When the cutoff value of Ki-67 was set to 14%, there was no difference according to Ki-67. RFS by Ki-67 had no statistical significance ($p = 0.416$; appendix 1) nor showed the difference between the low and high Ki-67 according to the PgR expression subsets ($p = 0.664$; appendix 2a, $p = 0.526$; appendix 2b). The best strategy is to use Ki-67 as a continuous marker, reflecting the biology of tumor proliferation. Moreover, treatment decision for individual patients should not depend on small differences of Ki-67 around a given cutoff point.
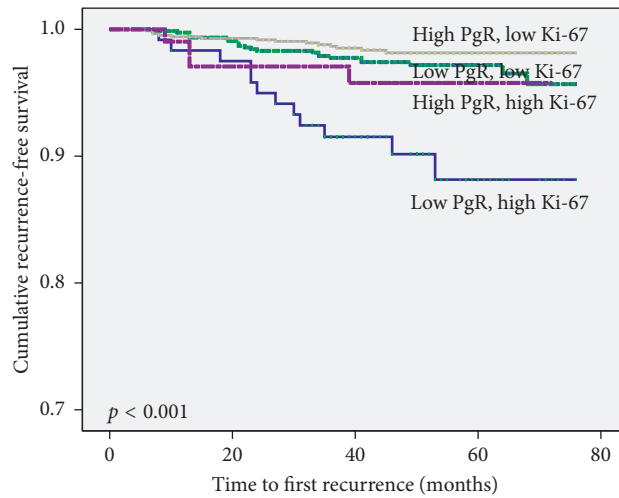
Figure 3: The recurrence-free survival of patients was divided into 4 group combination between Ki-67 and progesterone receptor expression.

Table 2: Multivariate Cox regression analysis for recurrence-free survival.

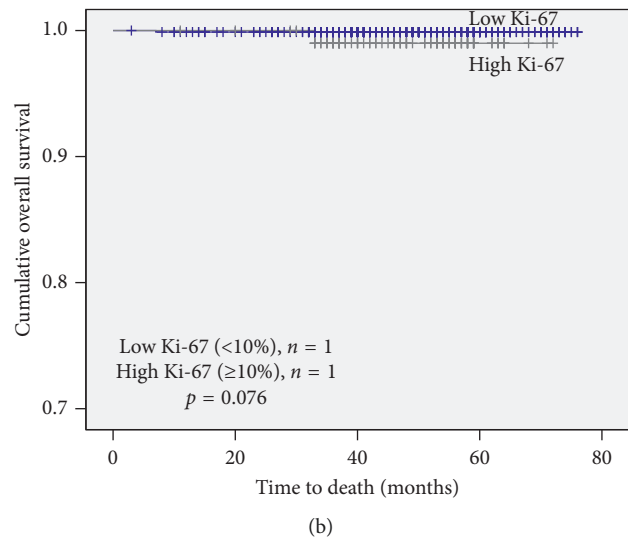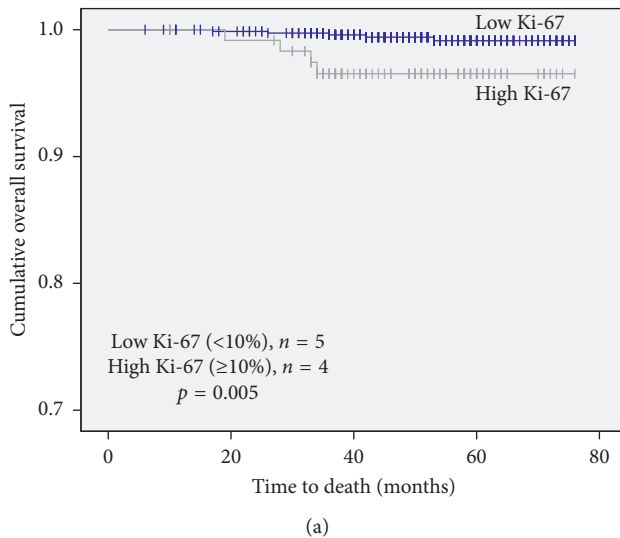|		Low PgR status		High PgR status	
		HR (95% CI)	p	HR (95% CI)	p
Age		1.00 (0.97–1.03)	0.856	0.99 (0.95–1.04)	0.713
Tumor size	≥2 cm, <2 cm	7.20 (2.53–20.51)	<0.001	5.40 (1.49–19.60)	0.010
Nodal status	Yes, no	1.66 (0.79–3.45)	0.179	2.53 (0.94–6.77)	0.065
Ki-67	≥10%, <10%	3.05 (1.50–6.19)	0.002	2.03 (0.61–6.72)	0.247
HG	High, low	1.78 (0.85–3.72)	0.127	0.82 (0.28–2.40)	0.719

(a)

(b)

Figure 4: Overall survival (OS) of patients in high and low Ki-67 breast cancer according to progesterone receptor expression status. (a) OS according to Ki-67 in the low progesterone receptor expression subset (PgR < 20%). (b) RFS according to Ki-67 in the high progesterone receptor expression subset (PgR ≥ 20%).

To our knowledge, this is one of the largest retrospective studies to analyze data from a high-quality clinical cancer registry on the routine use and prognostic significance of Ki-67. Although retrospective, it does have the advantage of comprising an unselected, nonmetastatic breast cancer population without selection bias. Notably, pathology and biomarker analyses were prospectively performed in a single, accredited laboratory, and thus represent a 'real' assessment of the value of IHC in clinical practice.

Despite the various limitations of Ki-67 as a marker, its clinical use in the breast cancer field has been adopted for several reasons. It is used to distinguish luminal tumors and

is considered a prognostic factor. Evaluating Ki-67 by IHC is inexpensive and easy to implement without investments in sophisticated equipment, leading to the attractive concept of Ki-67 as a low-cost biomarker. The importance of clinical flexibility is fundamental, given the uncertainties surrounding the tailoring of different chemotherapy options to each case, highlighting the importance of establishing a more accurate role for Ki-67.

In this study, we found that Ki-67 is an effective prognostic marker only in the context of low PgR status. We further found that patients with low PgR and high Ki-67 expression had the worst prognosis in terms of RFS. From a global perspective, genomic signatures will remain difficult to access in the foreseeable future for most patients. Active treatment can be considered as the default in cases of early ER-positive and HER2-negative breast cancer that satisfy conditions of low PgR and high Ki-67.

5. Conclusions

Our results show that Ki-67 has prognostic value for recurrence and survival in patients with ER-positive and HER2-negative early breast cancer only in the context of low PgR expression level. Thus, PgR expression should also be considered in evaluating the prognosis of breast cancer patients using Ki-67.

Data Availability

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

Conflicts of Interest

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

Supplementary Materials

Appendix 1: recurrence-free survival (RFS) according to the Ki-67 index by a 14% cutoff value. Appendix 2a: recurrence-free survival (RFS) according to Ki-67 (14% cutoff value) in the low progesterone receptor subset. Appendix 2b: RFS according to Ki-67 (14% cutoff value) in the high progesterone receptor subset. (*Supplementary Materials*)

References

[1] M. J. van de Vijver, Y. D. He, L. J. van't Veer et al., "A gene-expression signature as a predictor of survival in breast cancer," *New England Journal of Medicine*, vol. 347, no. 25, pp. 1999–2009, 2002.

[2] J. S. Parker, M. Mullins, M. C. U. Cheang et al., "Supervised risk predictor of breast cancer based on intrinsic subtypes," *Journal of Clinical Oncology*, vol. 27, no. 8, pp. 1160–1167, 2009.

[3] T. Sorlie, C. M. Perou, R. Tibshirani et al., "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications," *Proceedings of the National Academy of Sciences*, vol. 98, no. 19, pp. 10869–10874, 2001.

[4] S. Paik, S. Shak, G. Tang et al., "A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer," *New England Journal of Medicine*, vol. 351, no. 27, pp. 2817–2826, 2004.

[5] A. Goldhirsch, W. C. Wood, A. S. Coates et al., "Strategies for subtypes-dealing with the diversity of breast cancer: highlights of the st gallen international expert CONSENSUS on the primary therapy of early breast cancer 2011," *Annals of Oncology*, vol. 22, no. 8, pp. 1736–1747, 2011.

[6] T. O. Nielsen, F. D. Hsu, K. Jensen et al., "Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma," *Clinical Cancer Research*, vol. 10, no. 16, pp. 5367–5374, 2004.

[7] A. S. Coates, E. P. Winer, A. Goldhirsch et al., "Tailoring therapies-improving the management of early breast cancer: st gallen international expert Consensus on the primary therapy of early breast cancer 2015," *Annals of Oncology*, vol. 26, no. 8, pp. 1533–1546, 2015.

[8] NCCN Clinical Practice Guidelines in Oncology for Breast Cancer Version 1, 2017, http://wwwnccnorg/professionals/physician_gls/f_guidelinesasp.2017.

[9] A. Esposito, C. Criscitiello, and G. Curigliano, "Highlights from the 14(th) St Gallen International Breast Cancer Conference 2015 in Vienna: dealing with classification, prognostication, and prediction refinement to personalize the treatment of patients with early breast cancer," *Ecancermedicalscience*, vol. 9, p. 518, 2015.

[10] J. Chang, M. Ormerod, T. J. Powles, D. C. Allred, S. E. Ashley, and M. Dowsett, "Apoptosis and proliferation as predictors of chemotherapy response in patients with breast carcinoma," *Cancer*, vol. 89, no. 11, pp. 2145–2152, 2000.

[11] I. F. Faneyte, J. G. Schrama, J. L. Peterse, P. L. Remijnse, S. Rodenhuis, and M. J. van de Vijver, "Breast cancer response to neoadjuvant chemotherapy: predictive markers and relation with outcome," *British Journal of Cancer*, vol. 88, no. 3, pp. 406–412, 2003.

[12] M. Colozza, E. Azambuja, F. Cardoso, C. Sotiriou, D. Larsimont, and M. J. Piccart, "Proliferative markers as prognostic and predictive tools in early breast cancer: where are we now?," *Annals of Oncology*, vol. 16, no. 11, pp. 1723–1739, 2005.

[13] K. Tamaki, T. Moriya, Y. Sato et al., "Vasohibin-1 in human breast carcinoma: a potential negative feedback regulator of angiogenesis," *Cancer Science*, vol. 100, no. 1, pp. 88–94, 2009.

[14] H. Trihia, S. Murray, K. Price et al., "Ki-67 expression in breast carcinoma," *Cancer*, vol. 97, no. 5, pp. 1321–1331, 2003.

[15] F. Petrelli, G. Viale, M. Cabiddu, and S. Barni, "Prognostic value of different cut-off levels of Ki-67 in breast cancer: a systematic review and meta-analysis of 64,196 patients," *Breast Cancer Research and Treatment*, vol. 153, no. 3, pp. 477–491, 2015.

[16] E. de Azambuja, F. Cardoso, G. de Castro Jr., et al., "Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12 155 patients," *British Journal of Cancer*, vol. 96, no. 10, pp. 1504–1513, 2007.

[17] E. Luporsi, F. André, F. Spyratos et al., "Ki-67: level of evidence and methodological considerations for its role in the clinical management of breast cancer: analytical and critical review," *Breast Cancer Research and Treatment*, vol. 132, no. 3, pp. 895–915, 2012.

[18] T. O. Nielsen, J. S. Parker, S. Leung et al., "A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen

receptor-positive breast cancer," *Clinical Cancer Research*, vol. 16, no. 21, pp. 5222–5232, 2010.

[19] L. Harris, H. Fritsche, R. Mennel et al., "American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer," *Journal of Clinical Oncology*, vol. 25, no. 33, pp. 5287–5312, 2007.

[20] A. Goldhirsch, E. P. Winer, A. S. Coates et al., "Personalizing the treatment of women with early breast cancer: highlights of the st gallen international expert consensus on the primary therapy of early breast cancer 2013," *Annals of Oncology: Official Journal of the European Society for Medical Oncology*, vol. 24, no. 24, pp. 2206–2223, 2013.

[21] A. Prat, M. C. U. Cheang, M. Martín et al., "Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal A breast cancer," *Journal of Clinical Oncology*, vol. 31, no. 2, pp. 203–209, 2013.

[22] D. G. Tiezzi, J. M. Andrade, A. Ribeiro-Silva, F. E. Zola, H. R. Marana, and M. G. Tiezzi, "HER-2, p53, p21 and hormonal receptors proteins expression as predictive factors of response and prognosis in locally advanced breast cancer treated with neoadjuvant docetaxel plus epirubicin combination," *BMC Cancer*, vol. 7, p. 36, 2007.

[23] S.-Y. Jung, W. Han, J. W. Lee et al., "Ki-67 expression gives additional prognostic information on St. Gallen 2007 and Adjuvant! Online risk categories in early breast cancer," *Annals of Surgical Oncology*, vol. 16, no. 5, pp. 1112–1121, 2009.

[24] K. H. Allison, P. L. Kandalaft, C. M. Sitlani, S. M. Dintzis, and A. M. Gown, "Routine pathologic parameters can predict oncotype DXTM recurrence scores in subsets of ER positive patients: who does not always need testing?," *Breast Cancer Research and Treatment*, vol. 131, no. 2, pp. 413–424, 2012.

[25] S. S. Thakur, H. Li, A. M. Y. Chan et al., "The use of automated Ki67 analysis to predict oncotype DX risk-of-recurrence categories in early-stage breast cancer," *PLoS One*, vol. 13, no. 1, Article ID e0188983, 2018.

[26] M.-Y. C. Polley, S. C. Y. Leung, D. Gao et al., "An international study to increase concordance in Ki67 scoring," *Modern Pathology*, vol. 28, no. 6, pp. 778–786, 2015.

[27] M. C. U. Cheang, S. K. Chia, D. Voduc et al., "Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer," *JNCI: Journal of the National Cancer Institute*, vol. 101, no. 10, pp. 736–750, 2009.