

Prognostic relevance of DNA damage and repair biomarkers in elderly patients with hormone-receptor-positive breast cancer treated with neoadjuvant hormone therapy: evidence from the real-world setting

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Abstract

Background: The logic behind the outcome of endocrine therapy in breast cancer has long remained poorly understood. The prognostic role of DNA damage and repair biomarkers (DDR) was explored in postmenopausal, hormone-receptor-positive breast cancer patients treated with neoadjuvant hormone therapy (NAHT).

Methods: Data on 55 patients were included. The phosphorylated ataxia-teleangiectasia and Rad3-related protein (pATR), phosphorylated ataxia-telangiectasia mutated (ATM) kinase, and phosphorylated H2A Histone Family Member X (γ -H2AX) were evaluated by immunohistochemistry in paired tissues collected at baseline and following NAHT. Biomarkers were considered both singularly and within signatures. Ki-67 percentage change was the primary biomarker endpoint. Classical endpoints were also considered.

Results: The most favorable Ki-67 outcome was associated with the γ -H2AX/pATM signature ($p = 0.011$). In models of Ki-67 reduction, 'luminal B' subtype, higher grade of anaplasia, and the γ -H2AX/pATM signature tested as significant ($p < 0.05$ for all). Results were confirmed in multivariate analysis. No association was observed with pathologic response. An increase of $\Delta\gamma$ -H2AX in paired breast tissues was associated with longer event-free survival ($p = 0.027$) and overall survival ($p = 0.042$). In Cox models, both survival outcomes were solely affected by grade of anaplasia, with less favorable prognosis in the highest grades ($p < 0.05$ for both).

Conclusions: We report novel evidence of the prognostic role of DDR biomarkers on important patient outcomes in postmenopausal hormone-receptor-positive breast cancer patients treated with NAHT. If confirmed in future and adequately sized trials, our results may help inform therapeutic decisions and clarify underlying biological mechanisms.

Keywords: DNA damage and repair, elderly patients, hormone-receptor-positive breast cancer, neoadjuvant hormone therapy, prognostic biomarkers

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Introduction

The use of hormonal agents in the neoadjuvant setting for breast cancer was first hypothesized by Fisher and colleagues, who described more favorable survival outcomes in rats when chemotherapy (CT) and endocrine therapy were provided prior to the surgical removal of a mammary tumor.¹ Since then, the inherent body of knowledge has grown substantially. In the IMPACT (Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen) study, the use of anastrozole was compared with tamoxifen or their combination in 330 postmenopausal women with estrogen-receptor-positive (ER+) breast cancer. The primary biomarker endpoint was Ki-67, with the greatest Ki-67 suppression being observed in the anastrozole group.² This evidence paralleled the association between Ki-67 changes and disease-free survival that emerged from the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial in the adjuvant setting.³ Further support for the prognostic relevance of Ki-67 changes has subsequently come from a smaller study of 158 patients with ER+ primary disease.⁴ More recently, results from the PeriOperative Endocrine Therapy for Individualizing Care (POETIC) trial, wherein 4,486 patients were randomly assigned to receive 2-week nonsteroidal aromatase inhibitors (AIs) or no treatment before surgery, have failed in providing support for the efficacy of the treatment tested in terms of time to recurrence (TTR). However, Ki-67 assessment at baseline and at 2 weeks proved independent prognostic value and a prespecified 10% cut off was suggested for patients' risk stratification in decisions concerning therapeutic management in the adjuvant setting.^{5,6}

The DNA damage and repair (DDR) pathways act as a complex machinery with a key role in preserving genomic stability throughout DNA damage correction and cell elimination in case of unmanageable damage.⁷ We have repeatedly investigated the prognostic relevance of biomarkers of DDR in breast and other cancers with consistent results.⁸⁻¹² Recently, the particularly vivid interest of the scientific community toward determinants of endocrine resistance in ER+ breast cancer patients has been further reinforced by the introduction of cyclin-dependent kinase (CDK) 4/6 inhibitors in the metastatic setting.¹³⁻²⁰ This has also provided new hints for investigation to our research on DDR biomarkers in breast cancer, which we have now extended to a historical cohort of postmenopausal ER+ breast cancer patients not amenable to conservative breast surgery. In ER+ patients, estrogen

exposure translates into cellular proliferation through ER binding. Rapidly replicating cells may be facilitated in eluding canonical checkpoints and accumulating mutations.²¹ In such a scenario, the DDR machinery may play a particularly relevant role in affecting treatment outcomes and the related biomarkers may hold prognostic significance. We have thus explored the prognostic role of biomarkers of DDR in patients with ER+ disease treated with neoadjuvant hormone therapy (NAHT). The data herein analyzed are from a prior trial addressing surgical and long-term outcomes in 144 elderly postmenopausal patients with locally advanced, ER+ breast cancer treated with preoperative aromatase inhibitors (AIs) for whom Ki-67% expression data from presurgical biopsies and breast surgery were available.²²

Methods

Patients and setting

We herein present data from an observational study of 55 ER/progesterone receptor (PgR)+, stage I-III breast cancer patients diagnosed and treated with NAHT between January 2003 and December 2012. As previously mentioned, these patients were part of a wider cases series ($N = 144$) of postmenopausal ER+ breast cancer cases not amenable to conservative breast surgery. The inherent details on the methods applied were reported elsewhere.²² In brief, following NAHT with AIs, patients from the main study underwent mastectomy or conservative surgery, along with sentinel-node biopsy and/or axillary lymph-node dissection based on the surgical decision. Following surgery, all patients continued treatment with AIs. Whenever indicated, decisions concerning adjuvant CT with or without trastuzumab or radio therapy (RT) were taken in light of the individual patient risk of disease recurrence, as defined by widely known prognostic factors balanced against patient comorbidities. Generally, adjuvant breast RT was administered to patients who had undergone conservative surgery and to women who had been treated with mastectomy and whose cancer represented one or more of the following features: stage cT3, cN2 or cN3 at diagnosis or stage pN2 after surgery.^{22,23} With regard to the smaller subset of interest ($N = 55$), data on demographics and relevant patient- and disease-related features were made available, along with details on the treatment administered and related outcomes. Selected DDR kinases, that is,

the phosphorylated ataxia-telangiectasia and Rad3-related protein (ATR) and phosphorylated ataxia telangiectasia mutated (ATM) kinases, and DNA damage biomarker, that is, phosphorylated H2A Histone Family Member X (γ -H2AX) were evaluated by immunohistochemistry (IHC) in breast-tissue samples collected at baseline and in surgical specimens after NAHT. This study is primarily focused on the assessment of the prognostic relevance of these latter biomarkers. The primary endpoint was represented by changes in Ki-67 percent expression between paired breast-tissue samples from core biopsies and surgery. For the purpose of our study, we evaluated both qualitative (no *versus* yes) and quantitative changes (in percentage) related to Ki-67%, as emerged by the comparison between the samples collected at baseline and the surgical tissues. A 5% point reduction (5PT%) between the Ki-67 value at baseline and its surgical counterpart was chosen as the threshold for quantifying the reductions observed. The related variable was categorized according to two modalities, that is, Ki-67 reduction greater than 5PT% *versus* other. Secondarily, we aimed to assess the prognostic relevance of the biomarkers of interest against the following endpoints: (a) the presence and extent of residual tumor- or node-associated disease in the surgical specimen; and (b) survival endpoints, that is, event-free survival (EFS) and overall survival (OS). Pathologic complete response (pCR) was defined as the absence of invasive cancer within the breast and lymph node/s, based on extensive sampling, that is, at least 10 sections, 2–4 μ m in thickness, from 3 different regions of the initial tumor site, as proposed by Kuerer *et al.*²⁴ EFS was defined as the time between breast cancer diagnosis and disease recurrence or death, whichever came first. OS was intended as the time between breast cancer diagnosis and death. The satellite and main study were conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the cancer centers involved. This study was approved by the Central Ethical Board of the Lazio Region (Comitato Etico Centrale Regione Lazio) on 9th June 2015, Protocol number: CEC/532/15. Prior to any study-related procedure, a written informed consent was secured concerning patient inclusion, data collection and analysis, biological sample collection, storage and assessment. Our study is compliant with the REMARK guidelines, in that it provides relevant information concerning its design, underlying

hypothesis, characteristics of the included patients and collected specimens, assay methods, and statistical analysis.²⁵

Biomarker assessment

The IHC assessment was carried out in formalin-fixed paraffin-embedded breast-tissue samples. Immunoreactivity was evaluated by two investigators blinded to baseline patient characteristics and treatment outcomes. Discordant cases were reviewed by a third observer. Hormone receptor immunoreactivity was scored using the Allred system and considered positive when it was greater than 2.²⁶

For the biomarkers of interest, that is, pATR, pATM, γ -H2AX, the following antibodies were used: antiphospho-ATR (Ser 428; clone EPR2184) rabbit monoclonal antibody (MAB; Abcam) at the dilution of 1:100 (pH 6), antiphospho-ATM (Ser1981) (clone 7C10D8) mouse MAB (Rockland) at the dilution of 1:200 (pH 6), and anti-phospho-H2AX (Ser139; clone JBW301) mouse MAB (Upstate Biotechnology, Upstate Biotechnology, Inc. Fisher Scientific, part of Thermo Fisher Scientific) at the dilution of 1:500 (pH 8). Positive and negative cases were classified consistently with the method applied in previously conducted studies from our group.^{9,10} In brief, γ -H2AX was classified as high and low/negative using the median score of all tumors as the cut-off points. For DDR kinases, samples were considered positive when $\geq 10\%$ of the neoplastic cells showed a distinct moderate (2+) or strong (3+) nuclear immunoreactivity. A semiquantitative score was obtained by multiplying the staining intensity (from 0 to 3) by the percentage of nuclear-expressing tumor cells (from 0 to 100). The score obtained could thus vary within a 0–300 range.

Statistical analysis

Descriptive statistics were computed for the variables related to clinical-pathologic features, treatment administered and related outcomes. Age was reported in terms of median and range, while crude numbers and percentages were used for categorical variables. The distribution of relevant disease- and treatment-related features by biomarker status (negative *versus* positive) was compared using Fisher's exact test or Chi-square test, depending on the number and size of the categories compared. The association between the previously

agreed upon endpoints and DDR biomarkers were considered both singularly and combined within signatures of potential prognostic relevance.

Logistic regression models were developed to test the impact of key disease- and patient-related features on time-independent endpoints, that is, Ki-67-related endpoints. Cox regression models were developed to evaluate the impact of clinical-pathologic characteristics on survival outcomes. Survival curves for EFS and OS were generated by the Kaplan-Meier product-limit method and compared using log-rank test. Statistical significance was set at $p < 0.05$. Statistical analyses were carried out using the SPSS software (SPSS version 21, SPSS Inc., Chicago, IL, USA).

Results

Figure 1 shows the most representative images related to the IHC staining of the biomarkers of interest in paired breast tissues, while descriptive characteristics of the study participants are reported in Table 1. Patients were all postmenopausal, with median age being 75.7 years, within a range of 56–88 years. In the vast majority of these women, the tumor mass showed a 2–5 cm diameter at baseline (cT2; $N = 44$; 80%), and revealed itself as an invasive ductal carcinoma at pathologic assessment in all cases but one ($N = 54$; 98.2%). ‘Luminal A’ and G1 were the most commonly represented molecular subtype and grade of anaplasia, respectively ($N = 43$; 78.2% and $N = 37$; 67.3%). ERs and PgRs were both expressed in 22 patients (40%), while ERs were singularly represented in the remaining cases. In four patients (7.3%), human epidermal growth-factor receptor 2 (HER2) was overexpressed or the related gene amplified. The percent expression of Ki-67 was less than 14 in 45 (81.8%) patients. Exemestane and letrozole were the most widely administered AIs ($N = 30$; 54.5% and $N = 22$; 40.0%, respectively). Twenty-two patients (40%) received NAHT for at least 6 months. As expected, the most common clinical responses were partial response (PR) and stable disease (SD), which occurred in 41 (74.5%) and 8 (14.5%) cases, respectively. In the adjuvant setting, all the patients received hormone therapy, while CT was administered to 13 patients. Among them, one (1.8%) was treated with an anthracycline-based regimen, eight (61.54%) received an anthracycline-taxane-free regimen, and in four (7.3%) both anthracyclines and taxanes were administered. Thirty (54.5%) patients also received adjuvant RT. At the time of data analysis, 10 cases of recurrence (18.2%) have been

detected, with 9 (90%) of these patients having developed distant metastases and 1 (10%) having shown local recurrence.

In Table 2, relevant clinic-pathologic features, length and type of NAHT are compared across categories dependent upon biomarker status (negative *versus* positive). None of the differences observed reached the preset threshold of statistical significance. In Table 3, we report on the associations between Ki-67% changes and biomarker status. The biomarkers of interest were considered both singularly and within signatures, since functionally interconnected. The presence and extent of changes in Ki-67 was significantly affected exclusively by the γ -H2AX/pATM signature as assessed prior to NAHT and was associated with more favorable outcomes ($p = 0.011$). These results were confirmed and reinforced when addressing this same association by using a dual-modality- instead of a triple-modality-dependent variable ($p = 0.007$; Supplementary Table 1).

Results from regression models of factors associated with a Ki-67 reduction greater than 5PT% are shown in Table 4. In the developed models, such a reduction was significantly more common in patients whose cancer showed a ‘luminal B’ (*versus* ‘luminal A’) subtype, an intermediate or high grade of anaplasia (*versus* a low grade), and whose tumor stained positive for the γ -H2AX/pATM signature ($p = 0.008$, $p = 0.031$ and $p = 0.008$, respectively). All associations retained statistical significance when including all the cited factors ($p = 0.002$, $p = 0.02$, and $p = 0.004$, respectively).

At the time of pathologic assessment, no pCR cases were recorded. The extent of residual disease assessed in postsurgical samples was tested against the IHC status of the biomarkers of interest. The categorical variable included two modalities, that is, (a) residual disease in breast tumor tissue ($N = 29$) or lymph nodes ($N = 26$); and (b) residual disease in breast tumor **and** lymph nodes. No significant associations were observed between these biomarkers and response, either at all ($p = 0.831$), or across strata dependent upon Ki-67 reduction $> 5\%$ ($p = 0.397$; data available upon request).

In survival analysis of EFS, an increase of pre- to post-treatment changes (Δ) of γ -H2AX in paired breast tissues was associated with the highest percentage of patients free from relapse at 96 months of follow up ($p = 0.027$; Figure 2). When evaluating OS, $\Delta\gamma$ -H2AX affected OS at a fully statistically significant extent ($p = 0.042$; Figure 3).

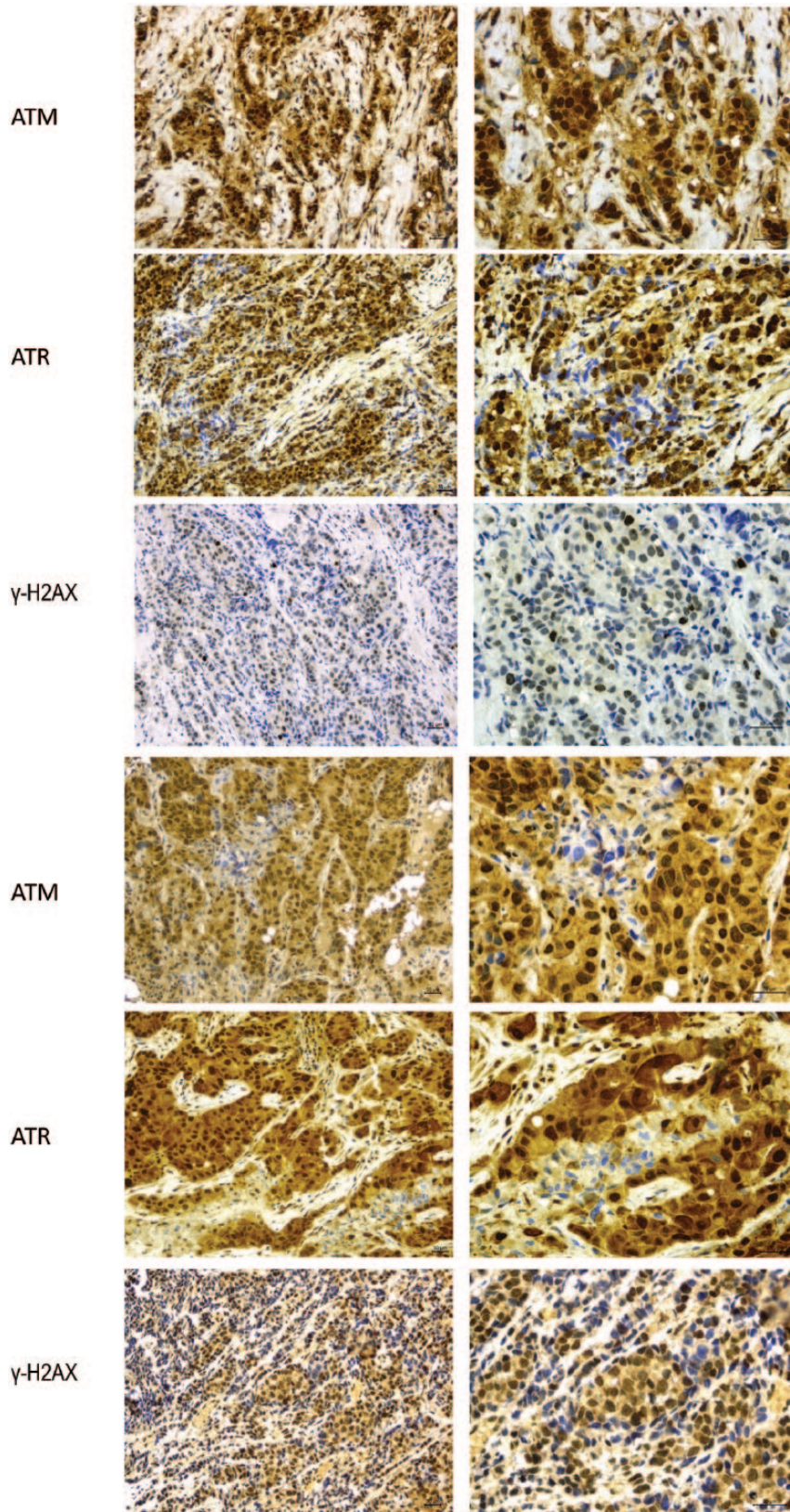


Figure 1. Representative examples of two breast cancer cases with pATR, pATM and γ -H2AX nuclear immunohistochemical expression.

Slide magnification in paired breast tissues at 20 \times (on the left) and 40 \times (on the right). Scale bar 30 μ m.

γ -H2AX, variant of histone H2AX phosphorylated in Ser139, histone; pATM, phosphorylated ataxia-teleangiectasia mutated; pATR, phosphorylated ataxia-teleangiectasia and Rad3-related protein.

Table 1. Baseline characteristics of the study participants ($N = 55$).

Characteristics		N (%)
Age at diagnosis	Median (min–max) [IQ range]	75.7 (56–88) [68.3–81.7]
cT	1	6 (10.9)
	2	44 (80.0)
	3	5 (9.1)
Histotype	Invasive ductal carcinoma	54 (98.2)
	Lobular carcinoma	1 (1.8)
Subtype	Luminal A	43 (78.2)
	Luminal B	12 (21.8)
Grade	G1	37 (67.3)
	G2	17 (30.9)
	G3	1 (1.8)
ER/PgR at the biopsy	ER+/PgR+	22 (40.0)
	Other+	33 (60.0)
HER2	Negative	51 (92.7)
	Positive	4 (7.3)
Ki-67	<14%	45 (81.8)
	≥14%	10 (18.2)
NAHT	Letrozole	22 (40.0)
	Anastrozole	3 (5.5)
	Exemestane	30 (54.5)
Duration of the NAHT	<6 months	33 (60.0)
	≥6 months	22 (40.0)
Clinical response	CR	4 (7.3)
	PR	41 (74.5)
	SD	8 (14.5)
	PD	2 (3.6)
Adjuvant CT	No therapy	42 (76.4)
	Anthracycline based	1 (1.8)
	Anthracycline–taxane free	8 (14.5)
	Anthracycline+taxane	4 (7.3)

Table 1. (Continued)

Characteristics		N (%)
Adjuvant RT	No	25 (45.5)
	Yes	30 (54.5)
Relapse	No	45 (81.8)
	Yes	10 (18.2)
	Local	1 (10.0)
	Distant	9 (90.0)

cT, Clinically-instrumentally defined primitive tumor size; CT, chemotherapy; ER/PgR, estrogen receptor/progesterone receptor; HER2, human epidermal growth-factor receptor 2; IQ, interquartile; NAHT, neoadjuvant therapy; CR, complete response; PR, partial response; SD, stable disease; PD, disease progression; RT, radiotherapy.

Table 2. Association between the singular biomarkers of interest and clinical and pathological characteristics (N = 55).

		pATR		Fisher's exact test
		Negative	Positive	p value
		N (%)	N (%)	
Age at diagnosis	≤76 years	3 (10.7)	25 (89.3)	0.236
	>77 years	0 (0.0)	27 (100.0)	
Subtype	Luminal A	3 (7.0)	40 (93.0)	0.999
	Luminal B	0 (0.0)	12 (100.0)	
ER/PgR	other	3 (13.6)	19 (86.4)	0.059
	ER+/PgR+	0 (0.0)	33 (100.0)	
Grade	G1	2 (5.4)	35 (94.6)	0.999
	G2+G3	1 (5.6)	17 (94.4)	
NAHT	Letrozole/anastrozole	2 (8.0)	23 (92.0)	0.585
	Exemestane	1 (3.3)	29 (96.7)	
Duration of the NAHT	<6 months	1 (3.0)	32 (97.0)	0.557
	≥6 months	2 (9.1)	20 (90.9)	
		pATM		
		Negative	Positive	
		N (%)	N (%)	
Age at diagnosis	≤76 years	15 (53.6)	13 (46.4)	0.422
	>77 years	11 (40.7)	16 (59.3)	

(Continued)

Table 2. (Continued)

		pATM		
		Negative	Positive	
		N (%)	N (%)	
Subtype	Luminal A	19 (44.2)	24 (55.8)	0.517
	Luminal B	7 (58.3)	5 (41.7)	
ER/PgR	other	11 (50.0)	11 (50.0)	0.788
	ER+/PgR+	15 (45.5)	18 (54.5)	
Grade	G1	19 (51.4)	18 (48.6)	0.407
	G2+G3	7 (38.9)	11 (61.1)	
NAHT	Letrazole+anastrozole	11 (44.0)	14 (56.0)	0.788
	Exemestane	15 (50.0)	15 (50.0)	
Duration of the NAHT	<6 months	18 (54.5)	15 (45.5)	0.271
	≥6 months	8 (36.4)	14 (63.6)	
		γ-H2AX		
		Negative	Positive	
		N (%)	N (%)	
Age at diagnosis	≤76 years	18 (64.3)	10 (35.7)	0.785
	>77 years	16 (59.3)	11 (40.7)	
Subtype	Luminal A	29 (67.4)	14 (32.6)	0.177
	Luminal B	5 (41.7)	7 (58.3)	
ER/PgR	Other	13 (59.1)	9 (40.9)	0.782
	ER+/PgR+	21 (63.6)	12 (36.4)	
Grade	G1	25 (67.6)	12 (32.4)	0.246
	G2+G3	9 (50.0)	9 (50.0)	
NAHT	Letrazole+anastrozole	14 (56.0)	11 (44.0)	0.578
	Exemestane	20 (66.7)	10 (33.3)	
Duration of the NAHT	<6 months	18 (54.5)	15 (45.5)	0.258
	≥6 months	16 (72.7)	6 (27.3)	

ER, estrogen receptor; NAHT, neoadjuvant hormone therapy; pATR, phosphorylated ataxia telangiectasia and Rad3-related protein; pATM, phosphorylated ataxia telangiectasia mutated; PgR, progesterone receptor; γ-H2AX, phosphorylated histone H2AX.

Table 3. Association between the biomarkers tested and Ki-67 changes ($N = 55$).

		Ki-67 change			Chi ² test
		No change + reduction < 5PT%	Reduction > 5PT%	Increased	<i>p</i> value
		<i>N</i> (%)	<i>N</i> (%)	<i>N</i> (%)	
γ-H2AX	Negative	20 (58.8)	12 (35.3)	2 (5.9)	0.092
	Positive	6 (28.6)	13 (61.9)	2 (9.5)	
pATM	Negative	13 (50.0)	10 (38.5)	3 (11.5)	0.398
	Positive	13 (44.8)	15 (51.7)	1 (3.4)	
pATR	Negative	2 (66.7)	1 (33.3)	0 (0.0)	0.746
	Positive	24 (46.2)	24 (46.2)	4 (7.7)	
γ-H2AX/pATM	Other	24 (55.8)	15 (34.9)	4 (9.3)	0.011
	γ-H2AX+/pATM+	2 (16.7)	10 (83.3)	0 (0.0)	
γ-H2AX/pATR	other	20 (58.8)	12 (35.3)	2 (5.9)	0.092
	γ-H2AX+/pATR+	6 (28.6)	13 (61.9)	2 (9.5)	

5PT%, 5% point reduction; pATR, phosphorylated ataxia-teleangectasia and Rad3-related protein; pATM, phosphorylated ataxia-teleangectasia mutated.

Table 4. Uni- and multivariate regression models of factors associated with a Ki-67 reduction greater than 5PT%.

		Univariate logistic regression model		Multivariate logistic regression model	
		OR (95% CI)	<i>p</i> value	OR (95% CI)	<i>p</i> value
Age at diagnosis	>76 years versus ≤76 years	0.76 (0.28–2.11)	0.605	0.90 (0.22–3.68)	0.879
Subtype	Luminal B versus Luminal A	9.33 (1.81–48.24)	0.008	22.81 (3.18–163.50)	0.002
Grade	III–II versus I	3.69 (1.12–12.14)	0.031	6.60 (1.32–33.08)	0.022
γ-H2AX/pATM	γ-H2AX+/pATM+ versus other	9.33 (1.81–48.24)	0.008	17.46 (2.39–127.63)	0.005

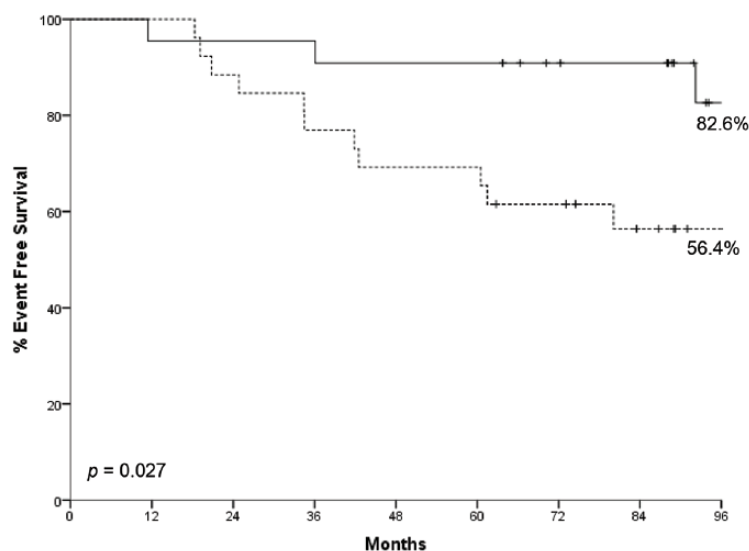
CI, confidence interval; 5PT%, 5% point reduction; γ-H2AX, variant of histone H2AX phosphorylated in Ser139, histon; OR, odds ratio; pATM, phosphorylated ataxia-teleangectasia mutated.

Factors having shown relevant impact on Ki-67 reduction in logistic regression models (Table 4) were further tested in univariate Cox models of EFS and OS. The only factor affecting survival outcomes significantly was the grade of anaplasia (G1 *versus* G2–G3, $p < 0.05$, for both the outcomes), with a less favorable prognosis being associated with the highest grades (data available upon

request). The results from univariate analyses and low number of events discouraged us from developing multivariate models.

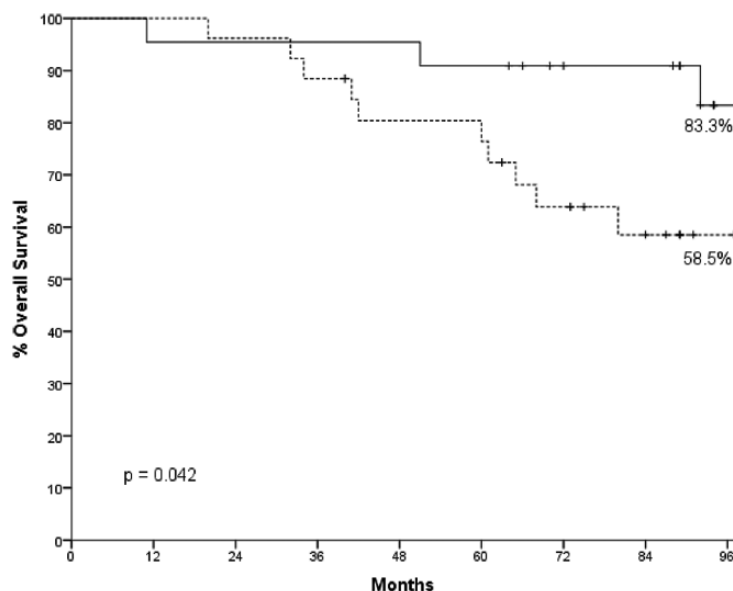
Discussion

We carried out an observational study focused on the prognostic relevance of selected biomarkers



Event-free survival analysis.
Kaplan–Meier curves showed that patients with an increase of the pre- to post-treatment changes (Δ) of γ -H2AX (solid line) in paired breast tissues had a longer time to relapse at 96 months of follow up compared with patients with a reduction/no modification of $\Delta\gamma$ -H2AX (dotted line; $p = 0.027$).

Figure 2. Event-free survival analysis by pre- to post-treatment changes (Δ) of γ -H2AX ($N = 55$).



Overall survival analysis.
The Kaplan–Meier curves showed that an increase of pre- to post-treatment changes (Δ) of γ -H2AX (solid line) in paired breast tissues was associated with a longer overall survival at 96 months compared with reduction/no modification of $\Delta\gamma$ -H2AX ($p = 0.042$).

Figure 3. Overall survival by pre- to post-treatment changes (Δ) of γ -H2AX ($N = 55$).

of DDR, that is, pATR, pATM, and γ -H2AX, in paired breast tissues collected from 55 patients treated with NAHT. When focusing on our study primary endpoint, that is, Ki-67 changes, we observed a first-time finding concerning

the significant association between the primary endpoint and one of the two signatures investigated. Patients whose tumor stained positive at baseline assessment for both γ -H2AX and pATM were significantly more likely to show a Ki-67

decrease greater than 5PT% in surgical tissues. This association was confirmed in univariate logistic regression models and retained its statistical significance when variables related to the molecular subtype, and grade of anaplasia were also included. None of the biomarkers tested influenced the extent of residual disease. In survival analysis, an increase of $\Delta\gamma\text{-H2AX}$ in paired breast cancer tissues was associated with a significantly higher percentage of patients free from relapse compared with patients for whom the comparison between bioptical and postsurgical tissues yielded a negative or decreased $\Delta\gamma\text{-H2AX}$.

In our study, Ki-67 changes were chosen as the primary endpoint. The opportunity for the design and conduct of trials of perioperative endocrine therapy with Ki-67 (being considered) as an intermediate marker of treatment benefit and long-term outcomes is based on the known effects of hormonal agents on cell proliferation, one of the main determinants of tumor growth.⁵ In recent years, three trials have paved the way to the study herein presented.^{2,4,6} Data from the IMPACT trial first substantiated the use of Ki-67 as a biomarker endpoint in ER+ breast cancers treated with NAHT.³ Results from a subsequent study from these same authors prompted evidence concerning the independent prognostic value of Ki-67 assessed not only at baseline, but also following 2 weeks of endocrine therapy.⁴ In the POETIC trial, the investigators did not observe any significant difference in the overall study population with regards to TTR ($p = 0.37$) or 5-year OS ($p = 0.83$). Conversely, when data were evaluated by baseline Ki-67, hazard ratios (HRs) and 95% confidence interval (CI) of TTR for Ki-67 greater than/equal to 10 were 2.6 (1.82–3.73, $p < 0.001$). In this same subset, a 2-week value of Ki-67 ≥ 10 identified a subgroup of patients whose HR and 95% CI for TTR were 2.2 (1.68–2.94, $p \leq 0.001$).⁶ Thus, Ki-67 changes helped identify high-risk groups within the overall study population. Our findings integrate this evidence to a further extent throughout the adjunctive assessment of biomarkers of DNA DDR. We observed first-time evidence of more favorable Ki-67 outcomes associated with the $\gamma\text{-H2AX/pATM}$ signature and, with regards to survival, an increase of $\Delta\gamma\text{-H2AX}$ in paired tissue samples. To our knowledge, no prior study has provided data on the prognostic value of this signature independently on the reduction of Ki-67, which represented our primary endpoint.

The activation of ATM through phosphorylation of Ser1981 (ATM -S1981P, pATM), and phosphorylation of histone H2AX at Ser 139 ($\gamma\text{-H2AX}$) are widely described not only as two among the main actors, but also as early biomarkers of cell response to DNA damage, particularly when this damage generates DNA double-strand breaks (DSBs).²⁷ The occurrence of DSBs triggers the DDR pathways, namely, pathways with a key role in DNA repair and cell cycle checkpoints, that arrest progression through the cell cycle to allow for DNA repair and, in the case of positive outcomes, resumption of DNA replication and cell division.^{12,28,29} ER+ breast cancer cells, that proliferate under estrogen exposure, may find it particularly difficult to tolerate large genomic rearrangements, which are more commonly associated with DSBs. This makes these cells more prone to succumb under the pressure of genotoxic insults, as well documented in studies of exposure to ionizing radiation and CT.^{30,31} When exposing ER+ breast cancer cell, which proliferate under estrogenic pressure, to NAHT, the observed association between biomarkers of DDR activation and more favorable outcomes following exposure to NAHT may represent an epiphenomenon of the sensitivity of these same cells to the proliferating stimulus provided by estrogen exposure. The aberrant activation of the DDR machinery, as expressed by the related biomarkers, may thus translate into a sort of functional hypertrophy of the machinery itself, which mirrors its reaction to the replicative stress induced by exposure to estrogens and, consequently, a plausible greater sensitivity to hormonal deprivation. Recent evidence from both the preclinical and clinical setting has also emerged and consistently supports the association between DDR defects and outcomes of endocrine therapy.^{32,33}

As previously cited, we have dealt with the prognostic role of biomarkers of DNA damage and response in prior work.^{8–12} Unfortunately, our results from these latter studies are barely comparable with those currently discussed in several respects. The most remarkable differences emerge when comparing these studies by cancer setting (advanced *versus* neoadjuvant),⁸ primitive cancer site other than breast,^{9–11} and exposure to CT rather than endocrine therapy in breast cancer patients from the same setting.^{10,12} In strict regard to these latter studies,^{10,12} the association we observed between some of the DDR biomarkers tested and less favorable outcomes following exposure to neoadjuvant CT may reflect a

strategy intrinsically developed by cancer cells in an attempt to adapt to replication stress and endogenous DNA damage.³⁴ This, in turn, renders cancer cells less susceptible to chemotherapy-induced DNA damage. In this respect, findings from the present study do not contradict those from previous work.

Our study has some limitations. The sample size is modest. However, the limitation in size should also be interpreted in light of the monocentric nature of our study with respect to the enrollment procedures, since all the study participants were recruited at one single cancer center. Limitation in numbers might possibly be counterbalanced by homogeneity in clinical-instrumental assessment and patients' management. We should also mention the single-level analysis conducted in our study population, in that our evaluation was exclusively based on the use of IHC techniques in breast cancer tissue samples. We are aware of the need of a multilevel analysis integrating genomic and transcriptomic profiling with data related to immunostaining from breast tissues.

Among our study strengths, the topic addressed is particularly relevant and timely to a research agenda, as efficaciously rendered by the work of Feng and coauthors, who have quite recently published on the added value of ATM expression in increasing the prognostic relevance of Ki-67 in a large cohort of ER+ breast cancer patients.³⁵ This latter study significantly differs from ours in terms of patients included, intervention administered, and methods applied. Yet, its findings are somewhat consistent with ours, in that the authors highlight the potentials of combining high ATM and low Ki-67 expression as prognostic determinants of survival, independently on broadly known factors such as tumor size, grade, and lymph-node status. If confirmed in further and adequately sized investigations, our effort may provide clues on patients' stratification in terms of risk of disease progression, thereby contributing to subsequent therapeutic decisions. In more detail, positive staining for the γ -H2AX/pATM signature may predict more favorable response in terms of Ki-67 reduction, and more favorable survival outcomes may be foreseen in patients whose presurgical tissue revealed positive staining for γ -H2AX. In these patients, therapeutic decisions in the adjuvant setting may not be oriented toward CT, particularly in elderly patients with relevant comorbidities, which may importantly restrict the use of the available therapeutic armamentarium.

Conclusion

In conclusion, within a previously established pipeline on the prognostic role of biomarkers of DDR in breast and other cancers, we carried out an observational study on a relatively restricted, though well characterized, set of ER+ breast cancer patients treated with NAHT. We provided first-time findings concerning the prognostic relevance of the γ -H2AX/pATM signature on Ki-67-related endpoints and of γ -H2AX on survival. Our research is in line with the current interest toward core components of the DDR pathways, which are currently investigated as members of a novel class of endocrine-resistant drivers. As previously mentioned, encouraging results are already available. The inherent mechanistic studies will help clarify the fundamental connections between the different actors, that is, ER expression, endocrine therapy, and DDR pathways. The evidence awaited will have enormous reflections in ER+ breast cancer treatment across different settings.

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Conflict of interest statement

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Supplemental material

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