original article

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An aCGH classifier derived from *BRCA1*-mutated breast cancer and benefit of high-dose platinum-based chemotherapy in HER2-negative breast cancer patients

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Background: Breast cancer cells deficient for *BRCA1* are hypersensitive to agents inducing DNA double-strand breaks (DSB), such as bifunctional alkylators and platinum agents. Earlier, we had developed a comparative genomic hybridisation (CGH) classifier based on *BRCA1*-mutated breast cancers. We hypothesised that this BRCA1-like^{CGH} classifier could also detect loss of function of *BRCA1* due to other causes besides mutations and, consequently, might predict sensitivity to DSB-inducing agents.

Patients and methods: We evaluated this classifier in stage III breast cancer patients, who had been randomly assigned between adjuvant high-dose platinum-based (HD-PB) chemotherapy, a DSB-inducing regimen, and conventional anthracycline-based chemotherapy. Additionally, we assessed *BRCA1* loss through mutation or promoter methylation and immunohistochemical basal-like status in the triple-negative subgroup (TN subgroup).

Results: We observed greater benefit from HD-PB chemotherapy versus conventional chemotherapy among patients with BRCA1-like^{CGH} tumours [41/230 = 18%, multivariate hazard ratio (HR) = 0.12, 95% confidence interval (CI) 0.04–0.43] compared with patients with non-BRCA1-like^{CGH} tumours (189/230 = 82%, HR = 0.78, 95% CI 0.50–1.20), with a significant difference (test for interaction $P = 0.006$). Similar results were obtained for overall survival (P interaction = 0.04) and when analyses were restricted to the TN subgroup. Sixty-three percent (20/32) of assessable BRCA1-like^{CGH} tumours harboured either a *BRCA1* mutation ($n = 8$) or *BRCA1* methylation ($n = 12$).

Conclusion: *BRCA1* loss as assessed by CGH analysis can identify patients with substantially improved outcome after adjuvant DSB-inducing chemotherapy when compared with standard anthracycline-based chemotherapy in our series.

Key words: array comparative genomic hybridisation, BRCA1, breast cancer, high-dose chemotherapy, platinum salt, predictive marker

introduction

Most evidence for benefit of adjuvant systemic treatment comes from large clinical trials carried out in the general breast cancer population [1]. However, these trials do not generally consider the molecular heterogeneity of breast cancers, which may be related to treatment benefit of individual patients. The disadvantage of these traditional trials can be best illustrated with the example of trastuzumab. Its efficacy among breast cancer patients with an amplification of the human epidermal growth factor receptor-2 (HER2-positive) would likely have been overlooked in analyses of the general population since a large percentage of breast cancers is HER2 negative and therefore does not benefit from trastuzumab. Several systemic treatments might therefore have been discarded in the past, although they may have been proven beneficial if tested in a predefined targeted population.

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Among these discarded agents are bifunctional alkylators and platinum salts, which are not commonly used, with the exception of cyclophosphamide (Endoxan; Baxter International, Deefield, IL, USA), due to their relatively high toxicity and low level of efficacy in unselected breast cancer patients [2–5]. These agents act via formation of DNA cross links resulting in DNA double-strand breaks (DSBs). Preclinical and clinical evidence has emerged that a possible target of DSB-inducing agents are tumours with a non-functional BRCA1 protein, such as tumours with *BRCA1* mutations [6–9]. *BRCA1*-mutated tumours showed hypersensitivity to these agents, which may be related to the role of *BRCA1* in homologous recombination, a conservative mechanism for error-free repair of DSBs. Absence of homologous recombination, such as in *BRCA1*-mutated tumours, prohibits error-free repair of DSBs, which is reported to lead to cell death [10].

Furthermore, defects in homologous recombination activate alternative more error-prone mechanisms such as non-homologous end joining, presumably leading to genomic instability [11–13]. *BRCA1*-loss-related instability can be visualised by array comparative genomic hybridisation (aCGH) showing characteristic copy number aberrations (CNAs) in defined genomic loci in a tumour [14–17].

We have previously developed an aCGH BRCA1-like classifier aimed to differentiate between *BRCA1*-mutated and sporadic breast cancers with reasonable accuracy based on their characteristic CNAs [17]. This test has been shown to have a relatively high sensitivity but a somewhat lower specificity for *BRCA1*-mutated tumours. We hypothesised that tumours testing "false positive" with the classifier could represent tumours with functional *BRCA1* loss due to other causes than mutations, such as *BRCA1* promoter methylation. If true, the BRCA1-like^{CGH} classifier would identify a larger fraction of breast cancer patients, who might benefit from DSB-inducing agents.

The aim of this study was to determine whether the BRCA1-like^{CGH} classifier was capable of identifying patients benefiting from DSB-inducing agents. For this purpose, we studied a representative sample of stage III HER2-negative breast cancer patients who had been randomly assigned between two treatment arms; high-dose, platinum-based, alkylating chemotherapy (HD-PB chemotherapy), which is a DSB-inducing regimen, and a standard anthracycline-based regimen (conventional chemotherapy) in a trial with long-term follow-up [18]. We restricted our analyses to HER2-negative patients, as in the pivotal study HER2-positive patients did not benefit from HD-PB chemotherapy [18]. Since patients in our study had been randomised, we could differentiate between selective HD-PB chemotherapy benefit and general chemotherapy benefit. Accordingly, we evaluated whether the effect of HD-PB chemotherapy on survival differed by BRCA1-like^{CGH} classification based on multivariate proportional hazards regression with an interaction term. To explore the biology of BRCA1-like^{CGH} classified tumours, we studied their association with other markers for *BRCA1* loss. We studied basal-like status defined by immunohistochemistry (IHC) since this had been associated with *BRCA1*-mutated breast cancers [19, 20]. Secondly, we assessed *BRCA1* promoter methylation, which has been reported as an alternative mechanism for reduced *BRCA1* expression in basal-like breast cancer [21, 22]. Lastly, *BRCA1* mutation status was determined. Since we found a strong association between the BRCA1-like^{CGH} classified tumours and triple-negative status and these markers have all been associated with triple negativity, we investigated them in the triple-negative subgroup (TN subgroup).

patients and methods

BRCA1-like^{CGH} classification

A BRCA1-like^{CGH} classifier, which calculates the probability of belonging to the *BRCA1*-mutated class, had previously been constructed (see supplemental Appendix B, available at *Annals of Oncology* online). We determined the optimal cut-off of the BRCA1-like^{CGH} probability score to identify breast cancer patients likely to benefit from DSB-inducing agents (for details, see supplemental Appendix B, available at *Annals of Oncology* online). For this purpose, we studied metastatic breast cancer (MBC) patients who had participated in phase II studies of HD-PB chemotherapy ($n = 39$, MBC series described in supplemental Appendix B, available at *Annals of Oncology* online) [23–25]. We carried out BRCA1-like^{CGH} class detection on each individual aCGH tumour profile, resulting in either a BRCA1-like^{CGH} or a non-BRCA1-like^{CGH} score.

patient selection

We studied stage III HER2-negative breast cancer patients from a large randomised controlled trial (RCT) carried out in the Netherlands between 1993 and 1999 in the adjuvant setting (stage III series). Eligibility criteria have been published previously [18] (see supplemental Appendix A, available at *Annals of Oncology* online). Patients were randomly assigned between conventional chemotherapy (5*FEC: 5-fluorouracil 500 mg/m^2, epirubicin 90 mg/m^2, cyclophosphamide 500 mg/m^2) and HD-PB chemotherapy (4*FEC, followed by 1*CTC: cyclophosphamide 6000 mg/m^2, thiotepa 480 mg/m^2 and carboplatin 1600 mg/m^2) [18].

Due to practical (financial) constraints, we did not evaluate all 621 HER2-negative breast cancer patients, but randomly selected 320 HER2-negative patients (320/621 HER2-negative cases, 51%). Patient samples were included in analyses if formalin-fixed paraffin-embedded (FFPE) primary tumour tissue consisting of more than 60% of tumour cells was available and if they had been treated per-protocol. Figure 1 summarises the flow of patients through the study. All trials described in this article were approved by the Institutional Review Board of the Netherlands Cancer Institute. This study was designed according to the REporting recommendations for tumor MArker prognostic studies (REMARK) guidelines [27] following the predictive marker trial design of 'Indirect assessment: Marker by treatment interaction design, test of interaction' as described by Sargent et al. [28].

comparative genomic hybridisation

Genomic DNA was extracted from FFPE primary tumours as previously described [29]. For 11 patients, only lymph node tissue containing primary tumour tissue, removed at first diagnosis, was available. Of 11 samples, DNA concentrations were too low for direct aCGH analysis and these samples were amplified with the *BioScore*™ Screening and Amplification Kit (42440, Enzo Life Sciences BVBA, Zandhoven, Belgium). Tumour DNA and reference DNA were labelled and hybridised as published previously (see supplemental Appendix A, available at *Annals of Oncology* online) [30]. To determine the quality of each CGH profile and to be able to compare experiments, we used a profile quality and hybridisation quality score (see supplemental Appendix A, available at *Annals of Oncology* online).

mutation and methylation analyses

We screened for 38 known *BRCA1* mutations using allelic discrimination and multiplex PCR accounting for 853 of 1166 *BRCA1* families (~73%) in

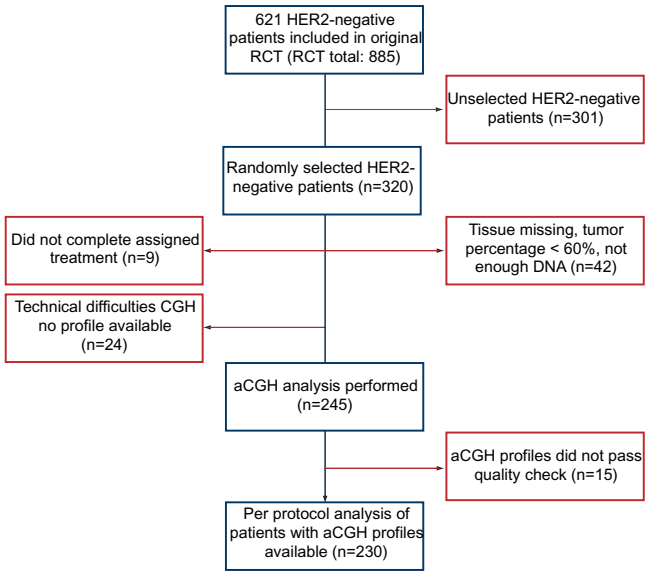


Figure 1. Flow diagram of patients in the study. Flow of patients through the study including number of patients in each stage. Reasons for dropout are listed. aCGH, array comparative genomic hybridisation.

the Netherlands (Supplemental Table S1, available at *Annals of Oncology* online) (M. K. Schmidt et al., unpublished data). Each putative mutation identified was validated using capillary sequencing.

Hypermethylation of the *BRCA1* promoter was assessed using a custom methylation specific MLPA set according to the manufacturer's protocol (ME005-custom; MRC-Holland, Amsterdam, the Netherlands). Probe sequences of the MLPA set are available on request (info@mlpa.com). DNA fragments were analysed on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For normalisation and analysis, the Coffalyzer program was used (MRC-Holland, Amsterdam, The Netherlands); peak heights below 250 were excluded from further analyses. When both *BRCA1* probes showed methylation (threshold of 0.2; MRC-Holland), we classified the result as *BRCA1* promoter methylation.

histopathology

Haematoxylin and eosin-stained slides were scored for tumour percentages. Estrogen receptor (ER), progesterone receptor (PR), P53 and HER2 status were determined by IHC as described previously [18, 32]. We used Pronase pretreatment for the epidermal growth factor receptor (EGFR Ab-10 clone 111.6; 1 : 200; Neomarkers / Lab Vision Corporation, Fremont, CA, USA; EGFR clone 31G7, 1 : 400; Zymed / Invitrogen, Carlsbad, CA, USA) and the standard procedure for cytokeratin 5/6 staining (CK5/6, clone D5/16 B4, M7237, 1 : 200; Dako, Glostrup, Denmark). CK5/6 and EGFR were considered positive if any staining of tumour cells was observed. Tumours were classified as basal-like according to the Nielsen basal-like breast cancer IHC definition [33].

statistical analysis

Differences between groups of interest were tested using Fisher's exact tests. Survival curves were generated using the Kaplan–Meier method and compared using log-rank tests. Hazard ratios (HR) were calculated using Cox proportional hazards regression.

Recurrence-free survival (RFS) was calculated from randomisation to appearance of local or regional recurrence, metastases or to death from any cause [18]. All other events were censored. Overall survival (OS) was time from randomisation to death from any cause or end of follow-up. Median RFS and OS were 7.6 and 8.2 years, respectively, for all 230 patients. Patients alive at last follow-up were censored at that time. All treatment comparisons were based on patients who completed their assigned treatment (per-protocol analysis) to secure the correct correlation between molecular subtype and treatment received. We assessed whether the effect of HD-PB chemotherapy versus conventional chemotherapy on survival, expressed as the HR, differed by BRCA1-like^{CGH} status based on multivariate proportional hazards regression with an interaction term, adjusting for potential confounders. All calculations were carried out using the statistical package SPSS 15.0.1 (for Windows) (SPSS Inc., Chicago, IL, USA).

results

Of the 320 randomly selected patients, 90 could not be analysed with aCGH due to unavailability or low quality of tumour tissue (i.e. tumour percentage, DNA yield, quality of DNA reflected by the aCGH quality score). In Figure 1, reasons for dropout are listed. Our selection held more ER- and PR-negative patients than the HER2-negative patients not selected for these analyses. Otherwise, characteristics and treatments of these 230 cases did not differ from those HER2-negative cases of the RCT not in current analyses (Supplemental Table S2, available at *Annals of Oncology* online).

Forty-one of the 230 tumours (18%) were scored as BRCA1 like^{CGH}. Patient characteristics did not differ by treatment arm within the BRCA1- or non-BRCA1-like^{CGH} subgroups (Table 1). When compared with patients with non-BRCA1-like^{CGH} tumours, patients with BRCA1-like^{CGH} tumours were generally younger and more often treated with breast-conserving surgery; their tumours were more often poorly differentiated, triple-negative, basal-like and P53-positive (Table 1).

outcome according to treatment in stage III series by BRCA1-like^{CGH} classification

The beneficial effect of HD-PB chemotherapy compared with conventional chemotherapy differed between patients with BRCA1-like^{CGH} tumours and those with non-BRCA1-like^{CGH} tumours (adjusted test for interaction $P = 0.006$). Among patients with BRCA1-like^{CGH} tumours, the risk of recurrence was eightfold decreased after HD-PB chemotherapy compared with conventional chemotherapy (adjusted HR 0.12, 95% CI 0.04–0.43; Table 2 and Figure 2B), while in patients with non-BRCA1-like^{CGH} tumours, no significant treatment difference was observed (adjusted HR 0.78, 95% CI 0.50–1.20; Table 2 and Figure 2A). Similar results were observed for OS (Figure 2C and D, adjusted test for interaction $P = 0.04$, data not shown). All analyses were adjusted for pathological tumour size, number of positive lymph nodes, Bloom–Richardson grade, triple-negative status and treatment as these were significantly associated with RFS (supplemental Table S3, available at *Annals of Oncology* online).

association of the BRCA1-like^{CGH} classifier within the triple-negative subgroup with BRCA1 mutation status, BRCA1 promoter methylation status and basal-like Nielsen phenotype

In the TN subgroup ($n = 60$), eight of the 13 *BRCA1*-mutated tumours had a BRCA1-like^{CGH} profile (Table 3). All 12 tumours with methylation of the *BRCA1* promoter displayed

Table 1. Patient characteristics distributed by treatment arm and BRCA1 classification of the stage III series

Variable	Patients with non-BRCA1-like^{CGH} tumours							Patients with BRCA1-like^{CGH} tumours							P^b
	Conventional chemotherapy		HD-PB chemotherapy		Total		P^a	Conventional chemotherapy		HD-PB chemotherapy		Total		P^a	
	N	%	*N*	%	*N*	%		*N*	%	*N*	%	*N*	%		
Total	95	50.3	94	49.7	189	100.0		23	56.1	18	43.9	41	100.0		n.s
Age in categories, years															
≤40	21	22.1	22	23.4	43	22.8	n.s	11	47.8	9	50.0	20	48.8	n.s	0.002
>40	74	77.9	72	76.6	146	77.2		12	52.2	9	50	21	51.2		
Type of surgery															
Breast-conserving therapy	16	16.8	18	19.1	34	18.0	n.s	8	34.8	6	33.3	14	34.1	n.s	0.03
Mastectomy	79	83.2	76	80.9	155	82.0		15	65.2	12	66.7	27	65.9		
Pathological tumour classification															
pT1 or pT2	80	84.2	78	83.0	158	83.6	n.s	19	82.6	17	94.4	36	87.8	n.s	n.s
pT3	15	15.8	14	14.9	29	15.3		4	17.4	1	5.6	5	12.2		
Unknown	0	0.0	2	2.1	2	1.1		0	0.0	0	0.0	0	0.0		
Number of positive lymph nodes															
4–9	66	69.5	59	62.8	125	66.1	n.s	15	65.2	11	61.1	26	63.4	n.s	n.s
≥10	29	30.5	35	37.2	64	33.9		8	34.8	7	38.9	15	36.6		
Histological grade															
I + II	63	66.3	63	67.0	126	66.7	n.s	4	17.4	1	5.6	5	12.2	n.s	<0.001
III	30	31.6	27	28.7	57	30.2		19	82.6	14	77.8	33	80.5		
Not determined	2	2.1	4	4.3	6	3.2		0	0.0	3	16.7	3	7.3		
Triple-negative status															
ER or PR positive (≥10%)	82	86.3	81	86.2	163	86.2	n.s	4	17.4	1	5.6	5	12.2	n.s	<0.001
Triple negative	13	13.7	13	13.8	26	13.8		18	78.3	16	88.9	34	82.9		
Unknown	0	0.0	0	0.0	0	0.0		1	4.3	1	5.6	2	4.9		
Nielsen basal-like breast cancer definition															
Negative	89	93.7	85	90.4	174	92.1	n.s	7	30.4	2	11.1	9	22.0	n.s	<0.001
Basal-like	6	6.3	9	9.6	15	7.9		15	65.2	15	83.3	30	73.2		
Unknown	0	0.0	0	0.0	0	0.0		1	4.3	1	5.6	2	4.9		
P53 status															
Negative (<10%)	51	53.7	65	69.1	116	61.4	0.05	8	34.8	7	38.9	15	36.6	n.s	0.02
Positive (≥10%)	40	42.1	26	27.7	66	34.9		12	52.2	8	44.4	20	48.8		
Unknown	4	4.2	3	3.2	7	3.7		3	13.0	3	16.7	6	14.6		

*P*atients with unknown values were omitted and *P* values were calculated using the Fisher's exact test.

[a]Association within subgroup.

[b]Association between subgroups.

CGH, comparative genomic hybridisation; ER, estrogen receptor; n.s., non-significant; PR, progesterone receptor.

a BRCA1-like^{CGH} profile (Table 3). All *BRCA1*-mutated tumours had an unmethylated *BRCA1* promoter. TN BRCA1-like^{CGH} tumours displayed in 88% (30/34), a basal-like phenotype. Conversely, 33% (15/45) of the basal-like tumours scored as non-BRCA1-like^{CGH} (Table 3). To explore the predictive potential of above markers and to put the BRCA1-like^{CGH} classifier in perspective, we assessed whether the effect of HD-PB chemotherapy on RFS differed by each separate marker with an interaction term.

outcome according to treatment in the triple-negative subgroup by different markers

Influence of the BRCA1-like^{CGH} classifier on differential treatment effect in the TN subgroup was similar to that observed in the total group of 230 patients (P interaction = 0.05). Subsequently, no substantial modification was seen of the HRs for RFS in BRCA1-like^{CGH} (adjusted HR: 0.17, 95% CI 0.05–0.60; Figure 2F and Table 4) and non-BRCA1-like^{CGH} patients (adjusted HR: 0.88, 95% CI 0.30–2.57; Figure 2E and Table 4). *BRCA1* methylation interacted significantly with the effect of HD-PB chemotherapy on RFS in the TN subgroup (interaction $P = 0.02$; Table 4). HD-PB chemotherapy effects differed less strongly by basal-like status or *BRCA1* mutation status and homogeneity was not rejected (P interaction: $P = 0.83$, $P = 0.76$, respectively, Table 4).

toxicity of HD-PB chemotherapy and marker status

There was no correlation between *BRCA1* status as assessed by mutation, methylation or aCGH analysis and early or late (non-)haematological toxicity of HD-PB chemotherapy.

discussion

In this study, we observed that a BRCA1-like^{CGH} classifier, derived from *BRCA1*-mutated tumours, was capable of selecting HER2-negative patients who had a significantly better outcome after HD-PB chemotherapy compared with conventional chemotherapy while there was no such evidence for unselected non-BRCA1-like^{CGH} patients (significant P interactions, RFS and OS). We found a similar high proportion

Table 2. Multivariate Cox proportional hazard analysis of the risk of recurrence (recurrence-free survival) in the stage III series

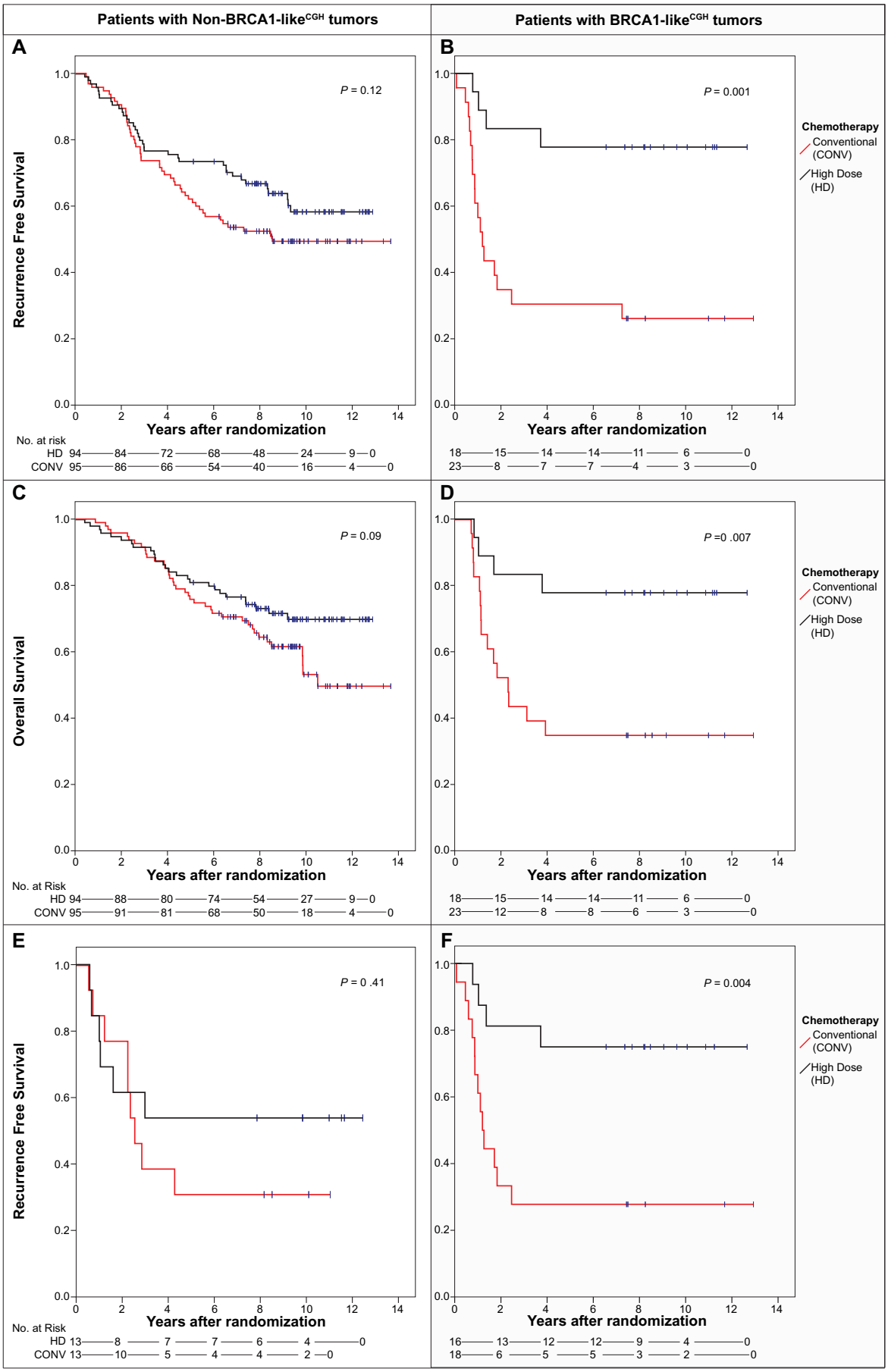
Variable	All patients stage III series			
	Number of events/number of patients	Hazard ratio	95% CI	P
Lymph nodes				
4–9	61/151	1.00		
≥10	43/79	1.71	1.13–2.59	0.01
p T-stage				
1 or 2	82/194	1.00		
3	22/34	1.95	1.19–3.22	0.009
Histological grade				
I + II	55/131	1.00		
III	47/90	1.54	0.98–2.40	n.s.
Hormone receptor status				
ER and PR negative (<10%)	32/60	1.00		
ER or PR positive (≥10%)	71/168	0.74	0.43–1.25	n.s.
aCGH classifier				
Non-BRCA1-like^{CGH} tumour	83/189	1.00		
BRCA1-like^{CGH} tumour	21/41	2.07	1.02–4.17	0.04
BRCA1-like^{CGH} tumour				
Conventional chemotherapy	17/23	1.00		
High-dose chemotherapy	4/18	0.12*	0.04–0.43	0.001
Non-BRCA1-like^{CGH} tumour				
Conventional chemotherapy	47/95	1.00		
High-dose chemotherapy	36/94	0.78*	0.50–1.20	n.s.

Homogeneity of both hazard ratios was rejected based on an interaction term with *P =0.006; Number of events is not equal for all variables since some patients have missing data; maximum missing variables (i.e. events) of all patients stage III series is 2/104 events.
aCGH, array comparative genomic hybridisation; CI, confidence interval; CGH, comparative genomic hybridisation; ER, estrogen receptor; n.s., non-significant; p, pathological; PR, progesterone receptor.

Table 3. Distribution of patients with a *BRCA1* mutation, a *BRCA1* methylation and basal-like status between BRCA1-like^{CGH} and non-BRCA1-like^{CGH} patients

Variable	Patients with non-BRCA1-like^{CGH} tumours		Patients with BRCA1-like^{CGH} tumours		P
	N	%	N	%	
BRCA1 mutation status[a]					
No mutation detected	19	73.1	26	76.5	n.s.
Mutation present	5[b]	19.2	8	23.5	
Undetermined	2	7.7	0	0.0	
BRCA1-promoter methylation status[a]					
Unmethylated	25	96.2	20	58.8	0.001
Methylated	0	0.0	12	35.3	
Undetermined	1	3.8	2	5.9	
Nielsen basal-like breast cancer definition[a]					
Negative	11	42.3	4	11.8	0.01
Basal-like	15	57.7	30	88.2	

[a]Analyses carried out in the triple-negative subset of the stage III series (n = 60). In seven BRCA1-like^{CGH} tumours, only ~62% of the types of *BRCA1* mutations prevalent in the Netherlands were determined due to technical difficulties instead of the intended ~73%. Similarly, of one non-BRCA1-like^{CGH} tumours ~40% of the type of *BRCA1* mutations could be tested.
[b]One patient scored just below the predetermined threshold of 0.63 the BRCA1-like^{CGH} classifier (score: 0.61). BRCA1 mutations were not necessarily germ line mutations since we tested DNA derived from the tumours. In all undetermined cases, all DNA had been used for array comparative genomic hybridisation analysis and no additional analyses could be carried out. Patients with unknown values were omitted and P values were calculated using the Fisher's exact test.
CGH, comparative genomic hybridisation; n.s., non-significant.


Patients with Non-BRCA1-like^CGH tumors
Patients with BRCA1-like^CGH tumors
A
B
C
D
E
F
Recurrence Free Survival
Overall Survival
Years after randomization
P = 0.12
P = 0.001
P = 0.09
P =0 .007
P = 0 .41
P = 0.004
Chemotherapy
Conventional (CONV)
High Dose (HD)
No. at risk
HD 94 84 72 68 48 24 9 0
CONV 95 86 66 54 40 16 4 0
No. at Risk
HD 94 88 80 74 54 27 9 0
CONV 95 91 81 68 50 18 4 0
HD 13 8 7 7 6 4 0
CONV 13 10 5 4 4 2 0
18 15 14 14 11 6 0
23 8 7 7 4 3 0
18 15 14 14 11 6 0
23 12 8 8 6 3 0
16 13 12 12 9 4 0
18 6 5 5 3 2 0

of triple-negative cases within BRCA1-like^{CGH} tumours (34/39, 87%) as in *BRCA1*-mutated tumours [34, 35] and therefore examined the classifier's association with *BRCA1* mutation, *BRCA1* methylation and basal-like status in the TN subgroup. We found that 63% (20/32) BRCA1-like^{CGH} tumours harboured either a *BRCA1* mutation ($n = 8$) or *BRCA1* methylation ($n = 12$), and these features were mutually exclusive. Furthermore, *BRCA1*-methylation status showed potential for the identification of patients with selective benefit of HD-PB chemotherapy; however, due to the small numbers, these data should be interpreted with caution and no conclusions can be drawn at this stage.

The BRCA1-like^{CGH} classifier displayed two characteristics required for efficacy in clinical practice. It selected a substantial number of patients (41/230). Secondly, in this series, it predicted a large differential treatment effect; selected patients showed an improved outcome after HD-PB chemotherapy when compared with standard anthracycline-based adjuvant chemotherapy and, just as importantly, unselected patients did not seem to have any advantage over standard chemotherapy as demonstrated by their HRs being close to one. Furthermore, it showed a large overlap with the other markers, 8/13 *BRCA1*-mutated tumours scored as BRCA1 like^{CGH}. Why all *BRCA1*-mutated tumours did not score as BRCA1 like^{CGH} is a matter of speculation. In two cases, the tumour cell content was estimated to be below 60% at blinded repeat examination, which may have caused excess 'dilution' of the tumour DNA by normal DNA. In a third case, a BRCA1-like^{CGH} score of 0.61 was found, while 0.63 was the predetermined threshold for a BRCA1-like^{CGH} status. In tumours with a low tumour percentage or tumours scoring near the threshold, confirmation of the test results by *BRCA1* sequencing may be advisable. In addition, many BRCA1-like^{CGH} tumours had a basal-like phenotype based on the Nielsen definition [33] in our series (~75%). However, basal-like phenotype and BRCA1-like^{CGH} do not seem to be identical markers since a substantial amount, one-third (15/45), of the basal-like tumours scored as non-BRCA1-like^{CGH}. Of the *BRCA1*-methylated tumours, 12/12 scored as BRCA1 like^{CGH}; given the small numbers, it could well be that the accuracy of the BRCA1-like^{CGH} classifier for identifying *BRCA1*-methylated cases is overestimated. However, it should be noted that in our study one-third of the BRCA1-like^{CGH} tumours showed *BRCA1*-promoter methylation, supporting our hypothesis that the classifier also identifies patients with *BRCA1* loss conferred by causes other than mutations. This hypothesis was further strengthened by a recent publication with a similar approach, in which *BRCA1/2*-mutated ovarian cancers were used to develop a gene expression profile of BRCAness [36]. In this study, 20/70 sporadic ovarian cancer patients scored as having BRCAness and had a significantly longer disease-free survival after platinum agents [36].

Our study is in line with previous findings in which *BRCA1* methylation was associated with good response to a platinum agent in 28 triple-negative breast cancer (TNBC) patients in the neoadjuvant setting [37]. In that study using tumour response according to Miller–Payne criteria as a surrogate end point for outcome, 2/2 TNBC patients with a *BRCA1* mutation achieved a pathological complete remission (pCR) on conventionally dosed cisplatin [37]. Similarly, Byrski et al. [9] studied a cohort of 102 *BRCA1* mutation carriers from 16 hospitals who had received various chemotherapy regimens in the neoadjuvant setting. Ten of the 12 patients (83%) achieved pCR on cisplatin monotherapy, while only 11 of the 51 (22%) patients who had received an anthracycline-based regimen achieved pCR [9]. Byrski et al. [9] cautioned, however, that their study was an observational study and patients in the cisplatin group had smaller tumours, were more often node negative and none of them had received prior chemotherapy, making direct comparison among treatment groups difficult. We did not observe a greater beneficial effect of platinum-based HD-PB chemotherapy over conventional chemotherapy in *BRCA1*-mutated compared with non-*BRCA1*-mutated breast cancers in the context of a RCT, which might at least partly be caused by the small numbers. Furthermore, we studied survival data with a median follow-up time of 7 years as the end point, instead of response to neoadjuvant chemotherapy. Additionally, it has been suggested that mutation site in the *BRCA1* gene could influence sensitivity to these agents [38]. Similarly, secondary mutations restoring the *BRCA1* reading frame in *BRCA1*-mutated cancers could lead to resistance, as has been described for ovarian cancers [39]. The low incidence of *BRCA1*-mutated breast cancer will make it challenging to resolve these remaining questions.

We did not find a significantly different benefit of HD-PB chemotherapy over conventional chemotherapy between basal-IHC and non-basal-IHC patients within the TN subgroup. In contrast, Diallo-Danebrock et al. [40] found an improved outcome after high-dose chemotherapy compared with dose-dense chemotherapy in high-risk breast cancer patients with a basal-IHC phenotype. However, this was not studied in the TN subgroup of patients and high-dose chemotherapy used in this study did not include a platinum salt. It is important to dissect TNBC in at least two subgroups, as TNBC has been shown to derive substantial benefit from addition of taxanes [41, 42], while in preclinical studies, relative resistance against taxanes has been demonstrated for breast cancer cells lacking functional BRCA1 [6, 7]. We hypothesise therefore that BRCA1-like^{CGH} TNBC patients should receive DSB-inducing regimens, while non-BRCA1-like^{CGH} TNBC patients should

Figure 2. Association of BRCA1-like^{CGH} classification with outcome after high-dose platinum-based (HD-PB) chemotherapy and conventional chemotherapy in all patients of the stage III series and the triple-negative subgroup. Kaplan–Meier survival curves according to the BRCA1 classification of patients who had been randomly assigned between HD chemotherapy and conventional chemotherapy. (A) Recurrence-free survival of non-BRCA1-like^{CGH} HER2-negative patients. (B) Recurrence-free survival of BRCA1-like^{CGH} HER2-negative patients. (C) Overall survival of non-BRCA1-like^{CGH} HER2-negative patients. (D) Overall survival of BRCA1-like^{CGH} HER2-negative patients. (E) Recurrence-free survival of non-BRCA1-like^{CGH} 'triple-negative' patients. (F) Recurrence-free survival of BRCA1-like^{CGH} triple-negative patients. CGH, comparative genomic hybridisation.

Table 4. Multivariate Cox proportional hazard analysis of the risk of recurrence (recurrence-free survival) for multiple markers in the triple-negative subgroup

Variable[a]	Number of events/ number of patients	Hazard ratio	95% CI	*P*
Nielsen basal-like tumour				
Conventional chemotherapy	15/21	1.00		
High-dose chemotherapy	8/24	0.36*	0.14–0.94	0.04
Non-basal-like tumour				
Conventional chemotherapy	7/10	1.00		
High-dose chemotherapy	2/5	0.45*	0.09–2.30	n.s.
BRCA1-like^{CGH} tumour				
Conventional chemotherapy	13/18	1.00		
High-dose chemotherapy	4/16	0.17**	0.05–0.60	0.006
Non-BRCA1-like^{CGH} tumour				
Conventional chemotherapy	9/13	1.00		
High-dose chemotherapy	6/13	0.88**	0.30–2.57	n.s.
BRCA1-mutated tumour				
Conventional chemotherapy	3/6	1.00		
High-dose chemotherapy	3/7	0.48***	0.08–2.98	n.s.
No mutation found in tumour				
Conventional chemotherapy	19/25	1.00		
High-dose chemotherapy	6/20	0.35***	0.13–0.91	0.03
BRCA1-methylated tumour				
Conventional chemotherapy	6/7	1.00		
High-dose chemotherapy	0/5	0.00****	0–0.17[b]	<0.001[b]
Unmethylated tumour				
Conventional chemotherapy	15/23	1.00		
High-dose chemotherapy	9/22	0.55****	0.23–1.31	n.s.

[a]All analyses shown were adjusted for marker of interest, lymph node status, pathological T-stage and histological grade as in Table 2. Homogeneity of both hazard ratios was tested with an interaction term resulting in: *$P = 0.83$, **$P = 0.05$, ***$P = 0.76$, ****$P = 0.02$.

[b]The upper confidence bound is based on a model restricted to patients with methylated tumours because it could not be calculated in the model including methylated and unmethylated tumour patients. Number of events is not equal for all variables since some patients have missing data; maximum missing variables (i.e. events) is 2/32.

CGH, comparative genomic hybridisation; CI, confidence interval.

receive taxane-based regimens. A neoadjuvant study has been initiated to test this hypothesis (NCT01057069).

The resolution of the CGH platform used in our study was lower compared with newer commercially available platforms. Nevertheless, it is unlikely that findings based on low resolution disappear on high resolution. Moreover, as we tested an existing classifier developed several years ago, we were confined to using the same platform. A limitation of our study was that it consisted of an unplanned subgroup analysis in a RCT. However, the use of a RCT allowed us to determine whether the association between markers and improved survival was related to either selective sensitivity to high-dose platinum-based chemotherapy or to general chemotherapy sensitivity/ resistance.

Despite increased toxicity of HD-PB chemotherapy in the whole group, we did not observe a difference between patients with or without a BRCA1-like^{CGH}, *BRCA1*-methylated or *BRCA1*-mutated tumour. This corroborates with the synthetic lethality concept in which cells with a functional *BRCA1* protein maintain their homologous recombination function and are capable of repairing the DSBs induced by the HD-PB chemotherapy, including normal tissues of *BRCA1* mutation carriers that did not lose the wild-type allele. In an era where we have largely abandoned HD-PB chemotherapy as a toxic regimen with no survival benefit, it is tempting to disregard the potential of the predictive markers investigated in our study, especially given the controversy surrounding this subject [43–45]. However, RFS differences observed between HD-PB chemotherapy and conventional, anthracycline-based chemotherapy in the BRCA1-like^{CGH} are remarkable. Presumably, this difference observed is an overestimation of the actual effect and should be confirmed in other studies. Because of constraints of the trial, unfortunately, we could not determine whether the platinum-based DSB-inducing regimen would have resulted in a similar improved outcome had it been conventionally dosed. We can only speculate that given the molecular background of the aCGH classifier (derived from *BRCA1*-mutated tumours) the type of agents is mandatory, all causing DSBs in the DNA, and explains the beneficial effect of HD-PB chemotherapy. This is particularly interesting given the fact that, recently a far less toxic, new DSB-inducing agent has been introduced in the form of poly(ADP) (PARP)-ribose inhibitors, which has been shown to target *BRCA1*-mutated breast cancer[8, 46]. Therefore, it would be interesting to consider the subgroup identified by the aCGH classifier for studies with PARP inhibitors only or in

combination with alkylating or platinum agents. To assess the usefulness of these markers for prediction of PARP inhibitor benefit, we have initiated a small pilot study in patients treated with an olaparib-containing regimen in the metastatic setting.

In conclusion, our data suggest that the BRCA1-like^{CGH} classifier might be predictive for selective HD-PB chemotherapy benefit, a DSB-inducing regimen. However, what the role of *BRCA1* methylation, basal-like and *BRCA1*-mutation status is remains unclear due to small numbers. This is the first study in breast cancer patients in which all these markers were evaluated in the context of a RCT with long-term outcome. However, these findings do not justify the introduction of HD-PB chemotherapy as a standard treatment option for breast cancer patients with a BRCA1-like^{CGH} tumour. The use of the aCGH classifier as a predictive marker for HD-PB chemotherapy, but especially for other DSB-inducing regimens (such as other alkylators, preferably in combination with PARP inhibitors) and the additive value of additional biomarkers, such as *BRCA1* methylation, separately and in combination warrants further investigation and validation, preferably in prospective RCTs.

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Contributors: SCL and SR were responsible for the study design. MAV coordinated the study. MKS and EHvB developed the research methods used. LFAW, PMN and EHvB constructed the BRCA1-like^{CGH} classifier. MAV, EHL, FEF, JGS, MHo, EGEdV, HvT took part in data collection. JW and MJvdV carried out all histopathological analyses. MAV, EHL and MdB carried out all experiments, except the mutation analyses, which were carried out by SC. MAV, MHa and HvT carried out the data analysis. MAV, EHL, SCL, PMN, LFAW and MHa took part in data interpretation. MAV and SCL drafted the manuscript with substantial contributions from all other authors. All authors gave their approval for submission. No medical writers were involved in writing this manuscript.

disclosure

SCL, MAV and PMN are named inventors on a provisional patent application for the aCGH BRCA1-like^{CGH} classifier used in this study; above authors have declared no further conflicts. All other authors of this paper have declared no conflicts of interest.

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