

# Pathological complete response and survival according to the level of HER-2 amplification after trastuzumab-based neoadjuvant therapy for breast cancer

S Guiu<sup>\*1</sup>, M Gauthier<sup>2,3</sup>, B Coudert<sup>1</sup>, F Bonnetain<sup>2,3</sup>, L Favier<sup>1</sup>, S Ladoire<sup>1</sup>, H Tixier<sup>4</sup>, B Guiu<sup>5</sup>, F Penault-Llorca<sup>6</sup>, F Ettore<sup>7</sup>, P Fumoleau<sup>1</sup> and L Arnould<sup>8</sup>

<sup>1</sup>Department of Oncology, Georges-François Leclerc Cancer Center, F-21000 Dijon, France; <sup>2</sup>Biostatistics Unit, Georges-François Leclerc Cancer Center, F-21000 Dijon, France; <sup>3</sup>EA 4184 School of Medicine, F-21000 Dijon, France; <sup>4</sup>Department of Surgery, Georges-François Leclerc Cancer Center, F-21000 Dijon, France; <sup>5</sup>Department of Radiology, CHU (University Hospital), F-21079 Dijon, France; <sup>6</sup>Department of Pathology, Jean Perin Cancer Center, F-63011 Clermont-Ferrand, France; <sup>7</sup>Department of Pathology, Antoine-Lacassagne Cancer Center, F-06189 Nice, France; <sup>8</sup>Department of Pathology, Georges-François Leclerc Cancer Center, F-21000 Dijon, France

**BACKGROUND:** We analysed whether the level of human epidermal growth factor receptor-2 (HER-2) amplification significantly influenced either pathological complete response (pCR) or recurrence-free survival (RFS) and overall survival (OS) after trastuzumab-based neoadjuvant therapy.

**METHODS:** In all, 99 patients with an HER-2-amplified breast tumour treated with trastuzumab-based neoadjuvant therapy were included. Tumours were classified as low amplified (LA; 6–10 signals per nuclei) or highly amplified (HA; > 10 signals). Pathological response was assessed according to Chevallier's classification (pCR was defined as grade 1 or 2). Median follow-up lasted 46 months (6–83). Cox uni- and multivariate analyses were performed.

**RESULTS:** In all, 33 tumour samples were LA and 66 were HA. The pCR in HA tumours was significantly higher than in LA tumours (55% vs 24%,  $P = 0.005$ ), whereas no association was found between the pCR rate and tumour stage, grade or hormone receptor status. In multivariate analysis, the pathological nodal status ( $P = 0.005$ ) and adjuvant trastuzumab ( $P = 0.037$ ) were independently associated with RFS, whereas the level of HER-2 amplification nearly reached statistical significance ( $P = 0.057$ ). There was no significant difference between LA and HA tumours for OS ( $P = 0.22$ , log-rank).

**CONCLUSION:** The level of HER-2 gene amplification significantly influenced pCR but not RFS or OS in non-metastatic breast cancer treated with trastuzumab-based neoadjuvant therapy. However, RFS in patients with HA tumours tended to be shorter.

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The human epidermal growth factor receptor-2 (*HER-2*) gene is amplified in 10–26% of human breast cancers (Gown *et al*, 2008). *HER-2* gene amplification is associated with the over-expression of the HER-2 protein in >95% of cases (Wolff *et al*, 2007). Both HER-2 over-expression and *HER-2* gene amplification have been correlated with poor clinical outcome (Slamon *et al*, 1987; Kallioniemi *et al*, 1991; Press *et al*, 1997). The HER-2 status is also a strong predictor of a clinical benefit from HER-2-targeted therapy, such trastuzumab (Herceptin; Roche, Neuilly-sur-Seine, France), a humanised monoclonal antibody directed against the external domain of HER-2 protein (Yamauchi *et al*, 2001). Several randomised trials have proved the efficacy of trastuzumab in metastatic (Slamon *et al*, 2001; Marty *et al*, 2005) and adjuvant (Piccart-Gebhart *et al*, 2005; Romond *et al*, 2005; Joensuu *et al*,

2006; Smith *et al*, 2007) settings for HER-2-positive breast cancer in terms of response rate, recurrence rate and a decrease in mortality. In the neoadjuvant setting, trastuzumab in association with chemotherapy had also shown a clinical benefit in terms of pathological complete response (pCR; Van Pelt *et al*, 2003; Buzdar *et al*, 2005; Coudert *et al*, 2006, 2007; Gianni *et al*, 2007).

American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines recommend using either immunohistochemistry (IHC) assays for initial evaluation of HER-2 status followed by reflex testing by fluorescence *in situ* hybridisation (FISH) of some IHC categories or FISH in initial testing (Wolff *et al*, 2007). The level of over-expression of HER-2 protein with IHC assays is a known predictive factor of response to trastuzumab (Slamon *et al*, 2001) and we have previously shown a positive correlation between the level of HER-2 amplification assessed by FISH and the rate of pCR to trastuzumab-based neoadjuvant treatment (Arnould *et al*, 2007). However, the relationship between the level of HER-2 amplification and the outcome of patients given neoadjuvant trastuzumab remains unclear.

\*Correspondence: Dr S Guiu; E-mail: sguiu@cgfl.fr

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The aim of this study was to determine whether the level of *HER-2* gene amplification using FISH assays significantly influenced recurrence-free survival (RFS) and overall survival (OS) in non-metastatic breast cancer treated with trastuzumab-based neoadjuvant therapy.

## MATERIALS AND METHODS

### Patients

Breast biopsies from 116 patients, who had received neoadjuvant trastuzumab in combination with chemotherapy for locally *HER-2*-positive breast cancer were retrospectively collected from 19 centres in France. All of the patients provided written, informed consent for their tissue material and clinical data to be centrally collected and used for research purposes. This study was approved by our institutional review board.

The patients were aged from 26 to 76 years (mean, 46.6 years) and had histologically confirmed, unilateral, unicentric, non-metastatic, *HER-2*-positive (in IHC) invasive ductal breast carcinoma. Most of the patients were treated in the framework of two open-label phase II clinical trials: GETN(A)-1 ( $n=63$ ; Coudert *et al*, 2007) and TAXHER-S01 ( $n=21$ ; Coudert *et al*, 2006). The remaining patients ( $n=32$ ) had an equivalent preoperative regimen to that used in the TAXHER-S01 trial.

The 63 patients included in the GETN(A)-1 trial had received weekly neoadjuvant trastuzumab ( $4\text{ mg kg}^{-1}$  loading dose followed by  $2\text{ mg kg}^{-1}$ ) in combination with docetaxel ( $75\text{ mg m}^{-2}$ ) and carboplatin (area under the curve of six) every 3 weeks for six cycles. Adjuvant trastuzumab was also administered in responding patients. The 21 patients included in the TAXHER-S01 trial had received the same preoperative schedule of trastuzumab in association with docetaxel ( $100\text{ mg m}^{-2}$ ) every 3 weeks for six cycles, but no adjuvant trastuzumab was scheduled in this study. Additional patients ( $n=32$ ) received trastuzumab every 3 weeks ( $8\text{ mg kg}^{-1}$  loading dose followed by  $6\text{ mg kg}^{-1}$ ) instead of weekly trastuzumab (Leyland-Jones *et al*, 2003) and adjuvant trastuzumab was also administered.

In all patients, 3 weeks after the last administration of neoadjuvant trastuzumab, tumours were surgically removed and pCR was assessed according to Chevalliers' classification: pCR was defined as no evidence of carcinoma either in the breast or in the lymph nodes, without (grade 1) or with (grade 2) *in situ* carcinoma. In accordance with institutional practices, adjuvant hormone therapy in patients with hormone receptor-positive tumours and adjuvant radiotherapy were mandatory.

### HER-2 status

The 84 patients included in the GETN(A)-1 or TAXHER-S01 trials were initially tested IHC 3+ or 2+ for *HER-2* status. For all of the patients in this study, *HER-2* status was re-analyzed centrally using both IHC and FISH assays by an experienced pathologist who was blinded to patient information, including the original IHC test results.

*HER-2* status in IHC was evaluated with A485 polyclonal antibody (Dako, Glostrup, Denmark) or 4B5 monoclonal antibody (Ventana Medical Systems Inc., Tucson, AZ, USA) on the BenchMark XT system (Ventana Medical Systems Inc.): biopsies were graded according to the HercepTest (Dako) scoring system (0+, 1+, 2+, or 3+).

FISH analyses were carried out using the *HER-2* Probe (Oncor, Gaithersburg, MD, USA) and BenchMark XT system. For each biopsy, *HER-2* signals were counted in  $\geq 60$  tumour cell nuclei and the mean *HER-2* signals per nuclei was calculated. The level of *HER-2* amplification in tumours was classified as follows: no amplified (NA; mean,  $< 6$  signals per nuclei), low amplified (LA; mean, 6–10 signals per nuclei), or highly amplified (HA; mean,

$> 10$  signals per nuclei or uncountable because of clusters of signals). The cutoff of 10 gene copies per nuclei to distinguish between LA and HA was chosen because it is the same as that proposed with chromogenic *in situ* hybridisation and also because above this cutoff, it is almost impossible to count signals precisely because of clusters and small aggregates. Borderline tumours (mean between four and eight signals per nuclei) were analysed by double-color FISH using a *HER-2*-gene-specific probe and a centromeric probe for chromosome 17 (PathVysion *HER-2* DNA Probe kit, Vysis-Abbott, Abbott Park, IL, USA) to determine *HER-2* amplification. In these cases, *HER-2* amplification was defined by a ratio of *HER-2* to chromosome 17 centromeric signals (*HER-2/CEP17*) of  $\geq 2.2$  (Wolff *et al*, 2007). All the tumours with  $\geq 6$  *HER-2* signals per nuclei had a *HER-2/CEP17* ratio  $\geq 2.2$  and therefore, were amplified tumours. All the tumours with  $< 6$  *HER-2* signals per nuclei had a *HER-2/CEP17* ratio  $\leq 1.8$  and thus, were NA tumours.

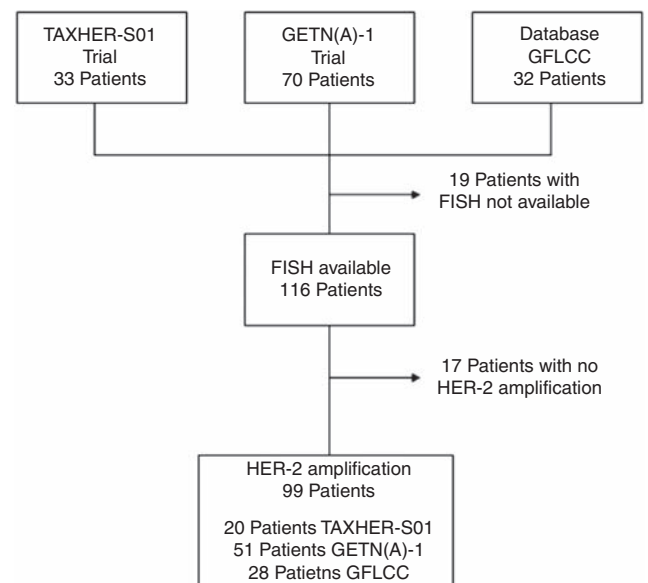
Only patients with centrally confirmed *HER-2* amplification were finally included in this study to evaluate pathological response rate (Figure 1). For RFS and OSs, NA tumours were also included.

### Statistical analysis

Qualitative variables were described using frequency and percentages.  $\chi^2$  and Fisher's exact tests were used to compare patient or tumour characteristics according to the level of *HER-2* amplification with FISH assays (NA, LA, and HA). For these analyses, Bonferroni adjustments were carried out to prevent inflation of type one error (the significant level was 0.016 for 3 comparisons).

Associations between tumour size, tumour grade, hormone-receptor status, level of *HER-2* amplification, and the presence or absence of pCR were evaluated using univariate and multivariate logistic regression. To take into account the trial effect (GETN(A)-1, TAXHER-S01, and GFLLC database), analyses were adjusted for this factor.

The median follow-up was calculated using the reverse Kaplan–Meier method. Recurrence-free survival was defined as the time from the date of histology to the date of the first recurrence of breast cancer at any site or death from any cause. Surviving patients without recurrence were censored at the last follow-up. The OS was defined as the time from the date of histology to death from any cause. Survival distributions were estimated with the



**Figure 1** Flow chart of the study. Abbreviations: GFLLCC, Georges-François Leclerc Cancer Center database; FISH, fluorescence *in situ* hybridisation

Kaplan–Meier method and compared using the log-rank statistic. Univariate (RFS and OS) and multivariate (RFS) Cox proportional hazards models stratified on the trial were fitted to test for an association between classical prognostic variables, the level of HER-2 amplification, pCR, adjuvant trastuzumab, and RFS or OS. Given the small number of events, multivariate analysis for OS was not performed. Akaike information criterion was computed for the goodness of fit for multivariate models and Harrell's *C*-statistic for discrimination (a Harrell's *C*-index = 0.5 indicates no predictive discrimination and a Harrell's *C*-index = 1.0 indicates perfect separation of patients) for each variable and for final multivariate Cox models. The multivariate models were internally validated using bootstrapping (100 replications). *P*-values were two-tailed and considered significant when less than 0.05. All analyses were performed using Stata V11 software (StataCorp LP, College Station, TX, USA).

## RESULTS

### Patients and tumours

Baseline patient and tumour characteristics are summarised in Table 1. In all, 99 (85%) tumours were considered amplified after central FISH analyses, among which 33 were classified as LA

tumours and 66 HA tumours. There were no significant differences between these two groups in terms of tumour stage, nodal status, hormone receptor status, or treatment given. The HA tumours had a higher histological grade than the LA tumours (*P* = 0.01).

### Analysis of pCR

According to Chevallier's classification (Table 2), 44 (44.5%) patients had a pCR, whereas 55 (55.5%) had no or only a partial response. In univariate logistic analysis, only the level of HER-2 amplification (FISH) was related to pCR (*P* = 0.005). In multivariate analysis, this variable was independently associated with pCR (*P* = 0.024), whatever the trial (*P* = 0.632).

### Recurrence-free survival according to HER-2 amplification

Median follow-up was 46-months (range, 6–83 months). Local or regional recurrences occurred in six patients (two HA tumours, two LA tumours, two NA tumours); one of these had a pCR. Metastatic recurrence (alone or with locoregional recurrence) occurred in 18 patients (14 HA tumours, 2 LA tumours, 2 NA tumours) among whom 5 had an initial pCR. Three patients died without a diagnosis of recurrent disease.

**Table 1** Patient and tumour characteristics according to the level of HER-2 amplification

Characteristic	Total (n = 116)	NA (FISH; n = 17)	LA (FISH; n = 33)	HA (FISH; n = 66)	<i>P</i> (3 groups; Fisher exact test)	<i>P</i> (NA vs LA/HA; Fisher exact test)	<i>p</i> (LA vs HA) (Fisher exact test)
Mean age (Range), year	46.6 (26.5–76.4)	46.6 (32–62)	48.5 (29–76.4)	45.6 (26.5–66)			
Tumour stage					0.699	0.474	0.710
T1	16 (14%)	3 (18%)	4 (12%)	9 (14%)			
T2	73 (63%)	12 (70%)	19 (58%)	42 (64%)			
T3–T4	27 (23%)	2 (12%)	10 (30%)	15 (22%)			
Nodal status					0.330	0.358	0.363
N0	55 (47%)	11 (65%)	12 (36%)	32 (49%)			
N1	59 (51%)	6 (35%)	20 (61%)	33 (50%)			
N2	2 (2%)	0	1 (3%)	1 (1%)			
Tumour grade					0.057	0.890	<b>0.010</b>
SBR1	5 (4%)	1 (6%)	0	4 (6%)			
SBR2	57 (49%)	9 (53%)	23 (70%)	25 (38%)			
SBR3	47 (41%)	6 (35%)	10 (30%)	31 (47%)			
Unknown	7 (6%)	1 (6%)	0	6 (9%)			
Hormone receptor status					0.072	<b>0.017</b>	0.782
Positive	68 (59%)	13 (76%)	20 (61%)	35 (53%)			
Negative	43 (37%)	2 (12%)	12 (36%)	29 (44%)			
Unknown	5 (4%)	2 (12%)	1 (3%)	2 (3%)			
Neoadjuvant treatment					0.315 <sup>a</sup>	0.145 <sup>a</sup>	0.670 <sup>a</sup>
TDC	63 (54%)	12 (70%)	18 (55%)	33 (50%)			
TD	53 (46%)	5 (30%)	15 (45%)	33 (50%)			
Central IHC score					<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>0.003</b>
1+	10 (8%)	10 (59%)	0	0			
2+	11 (10%)	6 (35%)	5 (15%)	0			
3+	95 (82%)	1 (6%)	28 (85%)	66 (100%)			
Pathological response					<b>&lt; 0.001<sup>a</sup></b>	<b>0.003<sup>a</sup></b>	<b>0.004<sup>a</sup></b>
pCR	45 (39%)	1 (6%)	8 (24%)	36 (55%)			
Non-pCR	71 (61%)	16 (94%)	25 (76%)	30 (45%)			
Adjuvant trastuzumab					0.769	0.557	0.872 <sup>a</sup>
No	32 (28%)	6 (35%)	9 (27%)	17 (26%)			
Yes	84 (72%)	11 (65%)	24 (72%)	49 (74%)			

Abbreviations: FISH = fluorescence *in situ* hybridization; HA = highly amplified tumours; IHC = immunohistochemistry; LA = low-amplified tumours; NA = no amplified tumours; non-pCR = absence of complete pathological response; pCR = pathological complete response; SBR = Scarff–Bloom–Richardson; TD = trastuzumab–docetaxel; TDC = trastuzumab–docetaxel–carboplatin. <sup>a</sup> $\chi^2$ -test. Values in bold: *P* < 0.05.

**Table 2** Univariate and multivariate logistic regression for predictive factors of pathological complete response

	pCR No/yes N = 99	Univariate analysis			pCR No/yes N = 92	Multivariate analysis			Bootstrapping <sup>a</sup>	
		OR	95% CI	P		OR	95% CI	P	95% CI	P
<i>Tumour stage</i>				0.714				0.537		0.691
T1	8/5	1	—		7/4	1				
T2	32/29	1.168	(0.311–4.387)		31/27	1.478	(0.290–7.529)		(0.009–228.220)	
T3–T4	15/10	0.781	(0.166–3.675)		14/9	0.791	(0.125–4.998)		(0.004–162.119)	
<i>Study</i>				0.570						
GETN(A)-I	28/23	1								
TAXHER-S01	13/7	0.602	(0.408–1.181)							
GFLCC	14/14	1.182	(0.725–1.464)							
<i>Tumour grade</i>				0.083				0.247		0.534
SBR1	3/1	1	—		3/1	1				
SBR2	32/16	1.476	(0.140–15.613)		31/16	2.407	(0.217–26.645)		(0–35 700 000)	
SBR3	18/23	3.987	(0.361–44.019)		18/23	4.761	(0.423–53.593)		(0–69 800 000)	
Unknown	2/4									
<i>Study</i>				0.706						
GETN(A)-I	28/23	1								
TAXHER-S01	13/7	1.043	(0.943–1.324)							
GFLCC	14/14	1.523	(0.423–1.544)							
<i>Hormone receptor</i>				0.299				0.166		0.250
Negative	20/21	1	—		19/21					
Positive	33/22	0.626	(0.271–1.445)		33/19	0.496	(0.184–1.338)		(0.150–1.638)	
Unknown	2/1									
<i>Study</i>				0.572						
GETN(A)-I	28/23	1								
TAXHER-S01	13/7	0.686	(0.500–1.230)							
GFLCC	14/14	1.302	(0.589–1.500)							
<i>Amplification (FISH)</i>				<b>0.005</b>				<b>0.024</b>		0.086
LA	25/8	1	—		24/8	1				
HA	30/36	3.862	(1.508–9.892)		28/32	3.190	(1.162–8.759)		(0.848–12.006)	
<i>Study</i>				0.520				0.632		0.631
GETN(A)-I	28/23	1			26/21	1				
TAXHER-S01	13/7	0.590	(0.354–1.193)		13/7	0.894	(0.220–3.632)		(0.181–4.412)	
GFLCC	14/14	1.188	(0.727–1.451)		13/12	1.584	(0.533–4.712)		(0.560–4.482)	

Abbreviations: CI = confidence interval; FISH = fluorescence *in situ* hybridisation; GFLCC = Georges–François Leclerc Cancer Center database; HA = highly amplified tumours; LA = low-amplified tumours; N = number; OR = odds ratio; pCR = pathological complete response; SBR = Scarff–Bloom–Richardson. <sup>a</sup>100 replications. Values in bold:  $P < 0.05$ .

RFS was not statistically different according to the pCR rate ( $P = 0.145$ , log-rank test; Figure 2A), or according to the HER-2 copy number ( $P = 0.313$ , log-rank test; Figure 2B) or the level of HER-2 amplification ( $P = 0.161$ , log-rank test). In the univariate Cox analysis, the level of HER-2 amplification was not significantly associated with RFS (Table 3), whereas the pathological nodal status (HR = 3.247 (CI 95%, 1.396–7.552),  $P = 0.006$ ) and adjuvant trastuzumab treatment (HR = 0.157 (CI 95%, 0.045–0.539),  $P = 0.003$ ) were. In multivariate analysis, pathological nodal status and adjuvant trastuzumab were independently associated with RFS, whereas the level of HER-2 amplification nearly reached statistical significance (HR = 2.819 (CI 95%, 0.970–8.197),  $P = 0.057$ ). Internal validation using bootstrapping confirmed the results only for the pathological nodal status.

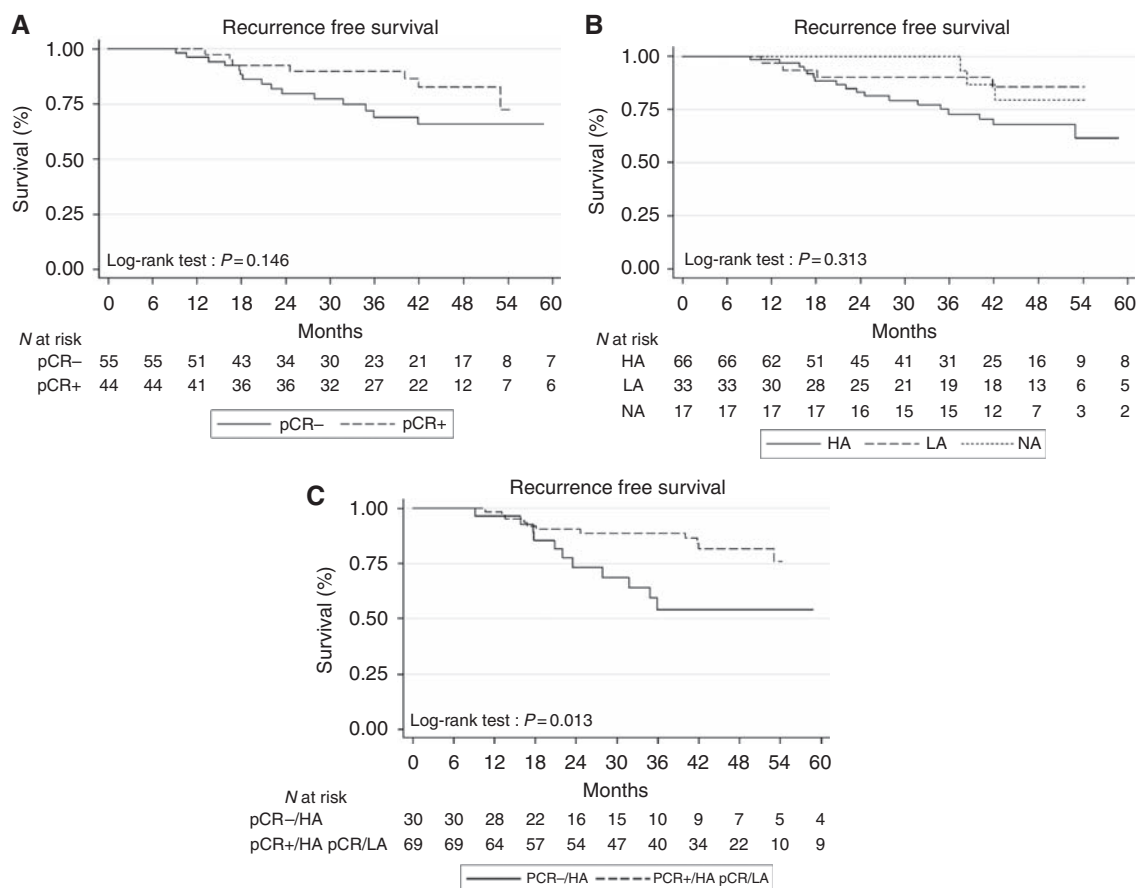
There was no significant difference in RFS according to both pCR and level of HER-2 amplification ( $P = 0.09$ , log-rank test). However, the subgroup of HA tumours without pCR had a significantly shorter RFS than did the other subgroups ( $P = 0.01$ , log-rank test; Figure 2C).

### Overall survival according to amplification of HER-2

During follow-up, 11 patients, including 8 with a metastatic recurrence (6 HA tumours, 2 LA tumours), died. There was no significant difference between NA, LA, and HA tumours subgroups for OS ( $P = 0.111$ , log-rank test; Figure 3) or between LA and HA tumours subgroups ( $P = 0.22$ , log-rank test). With Cox univariate analysis, only tumour stage (HR = 0.158 (CI 95%, 0.039–0.636),  $P = 0.034$ ) and pathological nodal status (HR = 7.118 (CI 95%, 1.864–27.177),  $P = 0.004$ ) were significantly associated with OS, whereas the level of HER-2 amplification was not (HR = 1.974 (CI 95%, 0.413–9.425),  $P = 0.394$ ).

### DISCUSSION

Systemic neoadjuvant therapy is the treatment of choice for locally advanced or inflammatory breast cancer. It also facilitates breast conservation in selected patients with operable disease (Kaufmann *et al*, 2006). For patients with HER-2-positive breast



**Figure 2** Recurrence-free survival according to the pathological response (**A**), the HER-2 copy number (**B**) and both pathological response and level of HER-2 amplification (**C**; Kaplan–Meier estimate). Abbreviations: HA, highly amplified tumours; LA, low-amplified tumours; NA, no amplified tumours; pCR+, pathological complete response; pCR-, absence of pathological complete response.

cancer (IHC 3+ and/or FISH positive), the addition of trastuzumab to chemotherapy increases pCR rates (Buzdar *et al*, 2005; Gianni *et al*, 2007). We confirm in this larger study our previous results (Arnould *et al*, 2007) regarding the positive correlation between the level of HER-2 amplification determined by FISH and the rate of pCR after trastuzumab-based neoadjuvant therapy. Indeed, we report 55 vs 24% ( $P=0.005$ ) of pCR in the subgroup of HA vs LA tumours, respectively. In light of these results, the level of HER-2 amplification could be a useful tool to decide whether to administer neoadjuvant therapy and could therefore also increase the rate of conservative surgery. However, this interesting predictive factor needs to be validated in further larger studies. Indeed, our results contrast with those of a smaller series in a neoadjuvant setting, in which response did not correlate with the level of HER-2 gene amplification measured by FISH (Buzdar *et al*, 2007); this study concerned 45 patients receiving paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide (FEC) with concurrent trastuzumab. Conversely, our results are consistent with those observed in 57 patients treated with trastuzumab plus chemotherapy (mainly paclitaxel) for a HER-2-positive metastatic breast cancer, in which a clinical objective response was significantly correlated with the level of amplification of HER-2 in FISH (Giuliani *et al*, 2007).

To date there are no data suggesting another mechanism than HER-2 gene amplification to explain the over-expression of the HER-2 protein. Consequently, both IHC and FISH can be used to determine HER-2 status and the benefit of trastuzumab in breast cancer (Wolff *et al*, 2007). In this study, after centralised analyses, 10 (8%) tumours were subsequently scored IHC 1+ (no pCR was

observed in this subgroup, data not shown). This highlights the modest inter-laboratory reproducibility of IHC results. This observation is in line with a recent critical review of the ASCO/CAP guidelines (Sauter *et al*, 2009), which concluded that inherent technical properties strongly argue for primary HER-2 FISH testing. Furthermore, although IHC and FISH have shown high concordance in some studies, reproducibility remains insufficient in others (Paik *et al*, 2002; Roche *et al*, 2002; Mass *et al*, 2005). In our study, 6 out of 11 (55%) tumours with an IHC score of 2+ were finally considered NA with the FISH assay (with only one pCR) and the patient with a 3+ tumour in IHC, which was NA in FISH did not benefit from trastuzumab (data not shown).

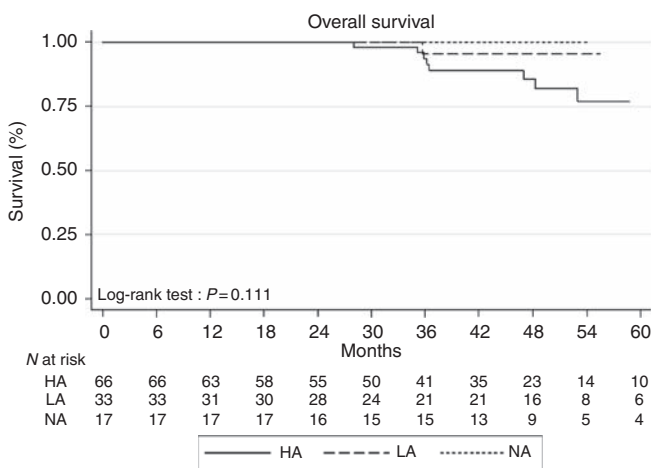
Currently, 52 weeks of adjuvant trastuzumab are recommended for the treatment of HER-2-positive breast cancer with a high risk of relapse. This regimen has improved both RFS and OS (Piccart-Gebhart *et al*, 2005; Romond *et al*, 2005; Smith *et al*, 2007; Slamon *et al*, 2009). Several trials in progress are comparing this standard with a shorter exposure to trastuzumab: 9 weeks as in the SOLD study (NCT00593697) and the Short HER study (NCT00629278) or 26 weeks (PHARE study). In a recently published study, a brief course of trastuzumab (9 weeks) administered concomitantly with docetaxel followed by three cycles of FEC tended to improve RFS but not OS, compared with the same regimen without trastuzumab (Joensuu *et al*, 2009). In our study, adjuvant trastuzumab, administered in addition to the 18 pre-operative injections, significantly improved RFS ( $P=0.003$ ). All these results are not in favour of lightening adjuvant trastuzumab. The above mentioned studies should resolve this question.



**Table 3** Univariate and multivariate Cox analysis of predictive factors of recurrence-free survival

	Recurrence or death No/yes, N = 99	Univariate analysis			Multivariate analysis			Bootstrapping <sup>a</sup>	
		HR	95% CI	P	HR, N = 98	95% CI	P	95% CI	P
<i>Tumour stage</i>				0.116			0.471		0.978
T1	7/6	1	—		1	—			
T2	47/14	0.446	(0.160–1.241)		0.642	(0.181–2.279)		(0.007–56.910)	
T3–T4	22/3	0.215	(0.048–0.972)		0.344	(0.062–1.911)		(0–6 160 872)	
<i>Tumour grade</i>				0.260					
SBR1	2/2	1	—						
SBR2	39/9	0.279	(0.058–1.351)						
SBR3	31/10	0.414	(0.082–2.076)						
Unknown	4/2								
<i>Hormone receptor</i>				0.175					
Negative	28/13	1	—						
Positive	46/9	0.547	(0.229–1.308)						
Unknown	2/1								
<i>Pathological nodal status</i>				<b>0.006</b>			<b>0.005</b>		<b>0.022</b>
Negative	59/12	1	—		1	—		(1.219–12.659)	
Positive	16/11	3.247	(1.396–7.552)		3.928	(1.524–10.127)			
Unknown	1/0								
<i>Amplification (FISH)</i>				0.199			0.057		0.883
LA	28/5	1	—		1	—			
HA	48/18	1.918	(0.710–5.180)		2.819	(0.970–8.197)		(0–2 667 202)	
<i>pCR</i>				0.161					
No	40/15	1	—						
Yes	36/8	0.523	(0.212–1.293)						
<i>pCR and FISH</i>				0.146					
No+HA	19/11	1	—						
No+LA	21/4	0.385	(0.120–1.228)						
Yes+HA	29/11	0.401	(0.148–1.090)						
Yes+LA	7/1	0.204	(0.025–1.669)						
<i>Adjuvant Trastuzumab</i>				<b>0.003</b>			<b>0.037</b>		0.779
No	14/12	1	—		1	—			
Yes	62/11	0.157	(0.045–0.539)		0.214	(0.050–0.914)		(0–10 456.280)	
Harrell's C-statistic					0.7745				
AIC					137				

Abbreviations: AIC = Akaike information criterion; CI = confidence interval; FISH = fluorescence *in situ* hybridisation; HA = highly amplified tumours; HR = hazard ratio; LA = low-amplified tumours; N = number; pCR = pathological complete response; SBR = Scarff–Bloom–Richardson. <sup>a</sup>100 replications. Values in bold:  $P < 0.05$ .



**Figure 3** Overall survival according to the HER-2 copy number (Kaplan–Meier estimate). Abbreviations: HA, highly amplified tumours; LA, low-amplified tumours; NA, no amplified tumours.

Pathological complete response is often considered a surrogate marker of outcome after neoadjuvant chemotherapy. Indeed, in several large trials with anthracycline and/or taxanes-based neoadjuvant therapy, RFS and OS rates were significantly improved when pCR had been achieved (Fisher *et al*, 1998; Kuerer *et al*, 1999; Guarneri *et al*, 2006). These studies were performed before the assessment of HER-2 status and the use of trastuzumab. In more recent studies with trastuzumab-based neoadjuvant therapy, the association between pCR and RFS has been inconclusive, sometimes statistically associated (Buzdar *et al*, 2007), but sometimes not (Hurley *et al*, 2006; Shimizu *et al*, 2009) as was the case in our study, despite a long follow-up. This prognostic factor thus remains controversial.

To our knowledge, this is the first study to report the outcome of patients after trastuzumab-based neoadjuvant therapy according to the level of HER-2 amplification in FISH. Although the increase in the number of HER-2 gene copies had a significant positive impact on the pCR rate, there was no significant difference between HA tumours (> 10 HER-2 gene copies per nuclei) and LA tumours (6–10 HER-2 gene copies per nuclei) for either RFS or OS after a median follow-up of 46 months. However, RFS tended to be

better in the HER-2 LA group ( $P=0.057$ ). A large population-based cohort, treated before the use of trastuzumab, had already shown that OS in breast cancer was not significantly different according to the level of HER-2 amplification, for patients with a HER-2/CEP17 ratio  $>2.2$  (Jensen *et al*, 2008). Since the era of trastuzumab therapy, the link between the level of HER-2 amplification and the outcome of patients has been investigated only in the metastatic and adjuvant settings. A retrospective analysis was performed in 33 patients with HER-2-positive metastatic breast cancer receiving trastuzumab (Gullo *et al*, 2009): patients with a high HER-2/CEP17 ratio had shorter time-to-progression and OS than did those with lower ratios (Gullo *et al*, 2009), although the difference did not reach statistical significance, probably because of the sample size and the relatively short follow-up. Dowsett *et al* (2009) analyzed whether the degree of HER-2 amplification (HER-2 gene copy number and HER-2/CEP17 ratio) influenced the clinical outcome in patients with HER-2-positive breast cancer randomised in the two HERA trial (Piccart-Gebhart *et al*, 2005) arms with or without 1 year of trastuzumab after adjuvant chemotherapy. Although there was an apparent trend

towards shorter disease-free survival with increasing HER-2 gene copy numbers or increasing HER-2 FISH ratios, the differences were not statistically significant. However, in this study, the median follow-up was only 2 years.

In conclusion, the level of HER-2 gene amplification using FISH assays significantly influenced pCR but neither RFS nor OS in non-metastatic breast cancer treated with trastuzumab-based neoadjuvant therapy. However, the subgroup of patients with HA tumours ( $>10$  signals per nuclei) tended to have a shorter RFS. This result suggests that a high level of HER-2 amplification could be a poor prognostic factor even though it was associated with a good initial sensitivity to trastuzumab. Further larger and longer studies are needed to confirm this hypothesis.

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## Conflict of interest

The authors declare no conflict of interest.

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