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Cyclophosphamide Dose Intensification May Circumvent Anthracycline Resistance of *p53* Mutant Breast Cancers

Jacqueline Lehmann-Che,[a,*] Fabrice André,[b,*] Christine Desmedt,[c] Chafika Mazouni,[d] Sylvie Giacchetti,[e] Elisabeth Turpin,[a] Marc Espié,[e] Louis-François Plassa,[a] Michel Marty,[e] Philippe Bertheau,[f] Christos Sotiriou,[c] Martine Piccart,[c] W. Fraser Symmans,[g] Lajos Pusztai,[g] Hugues de Thé[a]

[a]Biochemistry Department, Saint Louis Hospital, Assistance Publique Hôpitaux de Paris (AP/HP) and INSERM U944/CNRS UMR 7212/University Paris 7, Paris, France; [b]Institut Gustave Roussy, Villejuif, France; [c]Institut Jules Bordet, Bruxelles, Belgium; [d]Department of Surgery, IGR and Laboratoire de Transfert Biologique Oncologique, Marseille, France; [e]Maladies du Sein, Saint Louis Hospital, APHP, Paris, France; [f]Department of Pathology and INSERM U728/University Paris 7, St-Louis Hospital, APHP Paris, France; [g]MD Anderson Cancer Center, Houston, Texas, USA

Disclosures
Jacqueline Lehmann-Che: None; **Fabrice André:** None; **Christine Desmedt:** None; **Chafika Mazouni:** None; **Sylvie Giacchetti:** None; **Elisabeth Turpin:** None; **Marc Espié:** None; **Louis-François Plassa:** None; **Michel Marty:** None; **Philippe Bertheau:** None; **Christos Sotiriou:** None; **Martine Piccart:** None; **W. Fraser Symmans:** None; **Lajos Pusztai:** None; **Hugues de Thé:** None.

Section editor **Kathleen Pritchard** has disclosed no financial relationships relevant to the content of this article.

The content of this article has been reviewed by independent peer reviewers to ensure that it is balanced, objective, and free from commercial bias.

Learning Objectives

After completing this course, the reader will be able to:

1. Analyze the role of p53 mutation in ER-negative tumors in conferring increased sensitivity to high-dose alkylating agents, in order to treat patients with this phenotype using regimens containing high-dose alkylating agents.
2. Evaluate the role played by dysfunctional p53 in conferring chemosensitivity to anthracyclines, and explore the possibility of using high-dose alkylating agents to overcome the resistance of ER+/p53 mutated tumors.
3. Examine the mechanism for determining p53 gene function (functional analysis of separated alleles in yeast as opposed to immunohistochemistry) to more precisely determine the role of p53 activation in specific tumors, in order to select appropriate patients for treatment with high-dose alkylating agents.

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Abstract

The predictive value of p53 for the efficacy of front-line anthracycline-based chemotherapy regimens has been a matter of significant controversy. Anthracyclines are usually combined with widely different doses of alkylating agents, which may significantly modulate tumor response to these combinations. We analyzed three series

* Jacqueline Lehmann-Che and Fabrice André contributed equally to this work.
Correspondence: Hugues de Thé, M.D., Ph.D., INSERM/CNRS/University Paris 7, UMR 944/7212, Hôpital Saint Louis, 1, av Claude Vellefaux, 75 475 Paris Cedex 10, France. Telephone: 33-1-57-27-67-70; Fax: 33-1-57-27-67-95; e-mail: dethe@univ-paris-diderot.fr Received October 3, 2009; accepted for publication February 22, 2010; first published online in ***The Oncologist Express*** on March 12, 2010. ©AlphaMed Press 1083-7159/2010/ $30.00/0 doi: 10.1634/theoncologist.2009-0243

of de novo stage II–III breast cancer patients treated front line with anthracycline-based regimens of various cyclophosphamide dose intensities: 65 patients with estrogen receptor (ER)$^-$ tumors treated with anthracyclines alone (Institut Jules Bordet, Brussels), 51 unselected breast cancer patients treated with intermediate doses of cyclophosphamide (MD Anderson Cancer Center, Houston, TX), and 128 others treated with a dose-dense anthracycline–cyclophosphamide combination (St. Louis, Paris). After chemotherapy and surgery, pathologic complete response (pCR) was evaluated. p53 status was determined by a yeast functional assay on the pretreatment tumor sample. In a multivariate analysis of the pooled results, a lack of ER expression and high-dose cyclophosphamide administration were associated with a higher likelihood of pCR. A sharp statistical interaction was detected between p53 status and cyclophosphamide dose intensity. Indeed, when restricting our analysis to patients with ER$^-$ tumors, we confirmed that a mutant *p53* status was associated with anthracycline resistance, but found that p53 inactivation was required for response to the dose-intense alkylating regimen. The latter allowed very high levels of pCR in triple-negative tumors. Thus, our data strongly suggest that cyclophosphamide dose intensification in ER$^-$ *p53*-mutated breast cancer patients could significantly improve their response. ***The Oncologist*** 2010;15:246–252

Introduction

There is a need to identify drug-specific predictive biomarkers in order to better tailor chemotherapy regimens to individual patients with breast cancer. Several general biomarkers exist, including low estrogen receptor (ER) expression, high proliferative index, or high Onco*type* DX recurrence score, that could indicate general chemotherapy sensitivity in early-stage breast cancer [1]. However, there are no established drug-specific biomarkers, although several have been proposed, such as topoisomerase IIa amplification, which may predict the efficacy of anthracyclines [2], or low microtubule-associated protein τ (MAP tau) expression, which could predict paclitaxel efficacy [3].

In cell and animal models, p53 is the critical factor for the outcome of genotoxic stress, such as the one triggered by many cancer therapies [4–8]. Yet the role of *p53* mutations in predicting response to anthracylines or other chemotherapy drugs remains controversial. The frequency of *p53* mutations is around 30% in breast cancer patients in general [9]. The frequency of *p53* mutations is highly variable across breast cancer molecular subclasses. It is rare (0%–5%) in low-grade ER$^+$ breast cancer and can reach 95% in basal-like breast cancer [10]. Lack of p53 function precludes p53-triggered apoptosis or cell-cycle arrest [11, 12]. Some mutations can also exert dominant negative effects on p63 and p73, two related proteins with a key role in apoptosis and differentiation [13, 14]. Recent data have also implicated p53 in mammary stem cell fate determination. Collectively, *p53*-mutated tumors may not only have an altered response to cellular stress but also have an intrinsically distinct biology [15].

The relationship between *p53* mutations and chemotherapy efficacy has been extensively investigated, but no clear consensus has emerged. Some studies found that epirubicin has greater efficacy in patients with wild-type *p53* tumors [16–18], whereas other have observed that *p53* mutations are associated with much better efficacy of anthracycline-containing chemotherapy regimens [19, 20]. Technologies to assess *p53* mutations vary widely among studies and may not detect mutations with the same biological meaning. The distribution of molecular classes of breast cancer is not the same among studies and the predictive value of *p53* mutations could differ according to molecular class [20, 21]. The endpoint selected for evaluation of response also differs across studies—clinical response, pathological response, overall survival, or disease-free survival. Most importantly, widely different chemotherapy regimens have been administrated. In particular, widely different doses of alkylating agents have been combined with anthracyclines [16, 19, 20, 22].

Here, we analyzed the predictive value of p53 status for the efficacy of preoperative anthracycline-containing chemotherapy in three different series of breast cancer patients treated with increasing doses of cyclophosphamide. In patients treated with anthracyclines alone, we confirmed that a mutant *p53* status predicted resistance. Conversely, p53 inactivation was absolutely required for complete response to a dose-intense alkylating regimen. Thus, in the context of anthracycline therapy, p53 status is a critical biomarker for response to dose-intense cyclophosphamide.

Patients and Methods

Patients and Chemotherapy Regimens

Prospectively collected prechemotherapy specimens from patients with stage II–III breast cancer accrued to three different biomarker studies were included in the present analysis. In one study, conducted in the Saint Louis Hospital in Paris (SIM-HSL), 128 patients were treated with dose-dense epirubicin (75 mg/m^2) and cyclophosphamide (1,200 mg/m^2) (ddEC) administered every 15 days for six cycles. The efficacy and toxicity of this regimen were reported pre-

Table 1. Patient characteristics

Characteristic	Wild-type *p53* ($n = 99$)	Mutated *p53* ($n = 145$)	Overall ($n = 244$)
Median age (range), yrs	50 (23–76)	46 (27–78)	48 (23–78)
Stage			
T0–T2	20 (20%)	69 (48%)	89 (37%)
T3–T4	76 (77%)	76 (52%)	152 (62%)
Not assessable	3 (3%)	0	3 (1%)
Grade			
1–2	58 (59%)	27 (18%)	85 (35%)
3	27 (27%)	104 (72%)	131 (54%)
Not assessable	14 (14%)	14 (10%)	28 (11%)
ER status			
ER^+	62 (63%)	27 (19%)	89 (37%)
ER^-	20 (20%)	95 (65%)	115 (47%)
Not assessable	17 (17%)	23 (16%)	40 (16%)
HER-2 status			
HER-2 overexpressed or amplified	11 (11%)	26 (18%)	37 (15%)
HER-2 not overexpressed	55 (56%)	87 (60%)	142 (58%)
Not assessable	33 (33%)	32 (22%)	65 (27%)
Cyclophosphamide dose			
1,200 mg/m^2 every 2 wks (ddEC)	65 (66%)	63 (44%)	128 (52%)
500 mg/m^2 every 3 wks (FEC/FAC)	26 (26%)	25 (17%)	51 (21%)
No cyclophosphamide (E)	8 (8%)	57 (39%)	65 (27%)

Abbreviations: ddEC, dose-dense epirubicin and cyclophosphamide; E, single-agent epirubicin; ER, estrogen receptor; FAC, 5-fluorouracil, doxorubicin, and cyclophosphamide; FEC, 5-fluorouracil, epirubicin, and cyclophosphamide; HER, human epidermal growth factor receptor.

viously [23]. The second study cohort included 51 patients from the MD Anderson Cancer Center (MDACC) in Houston, Texas treated with 5-fluorouracil (500 mg/m^2), doxorubicin (50 mg/m^2), and cyclophosphamide (500 mg/m^2) (FAC) administered every 21 days for four cycles. The third series included 65 patients treated with single-agent epirubicin (E) (100 mg/m^2) given every 21 or 15 days for four or six cycles, respectively, in a clinical trial called TOP (Topoisomerase II alpha gene amplification and protein overexpression predicting efficacy of epirubicin) at the Institut Jules Bordet in Brussels. In contrast to the two other studies, that trial included only ER^- breast cancer patients.

Definition of Pathological Variables

ER status was determined by immunohistochemistry (IHC) (cutoff, >10% tumor cells stained positive). Human epidermal growth factor receptor (HER)-2 status was determined by IHC and/or fluorescence in situ hybridization (FISH) according to local procedures. HER-2 overexpression was defined as complete and intense membrane IHC staining in >10% of cells, or 30% of cells for patients treated in SIM-HSL, or an ErbB-2 to centromere 17 ratio >2 when FISH was performed. Grade was defined according to the Scarff–Bloom–Richardson criteria in the SIM-HSL series and TOP trial, and was defined as nuclear grade in the MDACC series. Pathologic complete remission (pCR) was defined as no invasive tumor cells in the primary tumor and axillary lymph nodes after chemotherapy.

p53 Determination

p53 gene functional status was determined by the functional analysis of separated alleles in yeast (FASAY) method [24], which evaluates the transactivation activity of p53 on a p53-responsive promoter stably integrated in the yeast genome. RNA was extracted from pretreatment tumor biopsies and reverse transcribed, and *p53* transcripts were amplified by polymerase chain reaction and transfected into yeast. Yeast colonies transformed with wild-type or mutated *p53* sequences appear as white and large or red and small, respectively. p53 status was considered mutated when: (a) >10% of the yeast colonies were red, (b) analysis using the split versions of the test could identify the defect in the 5′ or 3′ part of the gene, and (c) sequence analysis from mutant yeast colonies could identify an unambiguous genetic defect.

Table 2. Predictive parameters for chemotherapy efficacy

Characteristic	Univariate analysis OR (95% CI)	*p*-value	Multivariate analysis OR (95% CI)	*p*-value
Age (<50 versus ≥50)	0.96 (0.47–1.94)	.90	1.49 (0.57–3.85)	.42
Tumor size (cT3–cT4 versus cT1–cT2)	0.72 (0.35–1.47)	.37	0.71 (0.19–2.60)	.60
Grade (3 versus 1 or 2)	3.6 (1.4–9.1)	.007	1.18 (0.38–3.72)	.78
p53 (mutated versus wild type)	3.6 (1.5–8.5)	.004	3.4 (0.85–13.5)	.085
ER status (negative versus positive)	4.5 (1.8–11.3)	.002	12.2 (3.1–46.9)	<.001
Alkylating agent dose (ddEC versus other)	1.48 (0.73–2.9)	.28	10.9 (2.67–44.37)	.001
p53–alkylating agent interaction			6.4 (3.1–13.3)	<.001

Abbreviations: CI, confidence interval; ddEC, dose-dense epirubicin and cyclophosphamide; ER, estrogen receptor; OR, odds ratio.

Statistical Analyses

Associations between molecular and clinical variables and pCR were tested in a univariate analysis using a χ^2 or Fisher's exact test and in a multivariate logistic regression. All variables were included in a backward logistic regression model. Odds ratios (ORs), 95% confidence intervals (CIs), and *p*-values were estimated. Separate analyses were performed for all patients and for patients according to p53 and ER status. Patients with missing values for a particular marker were excluded from the corresponding analysis. A two-sided $p < .05$ was considered statistically significant. All statistical analyses were done using SPSS 12.0 software (SPSS, Inc., Chicago, IL).

Results

Patient Characteristics

The different clinicopathologic variables (ER, HER-2) and *p53* gene functional status (FASAY method) are presented in Table 1. Compared with a series of sporadic breast cancer cases, the present study was enriched in ER^- (56%, $n = 115$ of 204) and high-grade (61%, $n = 131$ of 216) breast cancers, at least in part because the TOP trial only included ER^- tumors. As expected, a much higher incidence of ER^- tumors was observed in *p53*-mutated tumors (78%, $n = 95$ of 122) than in wild-type *p53* tumors (24%, $n = 20$ of 82; $p < .001$). As in previous studies, *p53*-mutated tumors were more likely to present as high grade than wild-type *p53* tumors (79%, $n = 104$ of 131, versus 32%, $n = 27$ of 85; $p < .001$).

Predictive Parameters for Chemotherapy Efficacy

Chemotherapy response was directly assessed by pCR. In a univariate analysis pooling of all three trials (Table 2), high tumor grade (OR, 3.6; 95% CI, 1.4–9.1) and lack of ER expression (OR, 4.5; 95% CI, 1.8–11.3) were associated with a greater likelihood of pCR, in keeping with most previous studies [25]. p53 inactivation (OR, 3.6; 95% CI, 1.5–8.5) was also associated with a higher likelihood of pCR.

In contrast, on multivariate analysis (Table 2), although a lack of ER expression (OR, 12.2; 95% CI, 3.1–46.9; $p < .001$) remained highly significant (with a higher OR), grade and p53 status were no longer associated with response. Importantly, use of the dose-intense ddEC protocol was associated with a higher likelihood of pCR (OR, 10.9; 95% CI, 2.67–44.37; $p = .001$). These dramatic differences reflect the tight interconnection among several of the variables under study, including grade and p53 status, as well as cyclophosphamide dose intensity and ER status.

Predictive Value of p53 Inactivation for Efficacy of Increasing Doses of Cyclophosphamide

The predictive value of p53 inactivation for chemotherapy response may be intimately linked to the type of regimen. In that respect, a logistic regression model identified a very significant interaction between p53 status and efficacy of the high-dose alkylating agent (OR, 6.4; 95% CI, 3.1–13.3; interaction test $p < .001$) (Table 2). Indeed, the ORs for pCR and p53 status were highly heterogeneous across the three trials, because the test for heterogeneity yielded a value of 14.39 ($p = .0001$), implying that the predictive value of p53 for pCR is tightly linked to the type of regimen. As shown in Figure 1A, the pCR rates were 36% ($n = 23$ of 63), 4% ($n = 1$ of 25), and 12% ($n = 7$ of 57) in patients with *p53*-mutated tumors treated with high-dose (ddEC), standard-dose (FAC), or no (E) cyclophosphamide. Conversely, in patients with wild-type *p53* tumors (Fig. 1B), the pCR rates were 0% ($n = 0$ of 65), 15% ($n = 4$ of 26), and 37% ($n = 3$ of 8), respectively. Thus, dose intensification is associated with a much higher rate of complete response in patients with *p53*-mutated tumors.

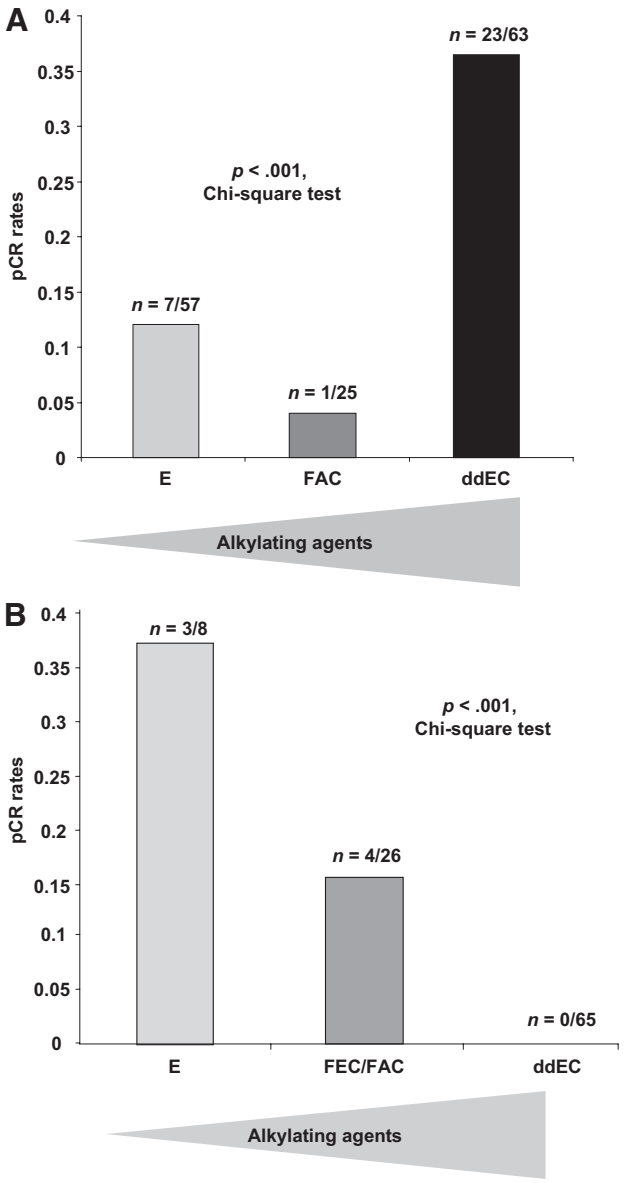

Figure 1. Pathologic complete response (pCR) rate according to alkylating agent dose. **(A):** Patients with *p53*-mutated tumors. **(B):** Patients with wild-type *p53* tumors.

Abbreviations: ddEC, dose-dense epirubicin and cyclophosphamide; E, single-agent epirubicin; FAC, 5-fluorouracil, doxorubicin, and cyclophosphamide.

Predictive Value of *p53* in ER$^-$ Disease

Because one of the trials only recruited patients with ER$^-$ tumors, an imbalance in ER expression according to p53 status might have biased our results. We thus compared the efficacies of the three regimens in a more homogeneous subgroup of patients with ER− *p53*-mutated tumors. The latter is highly enriched in basal breast cancers [10], although it likely also contains some molecular apocrine ones [26, 27]. In patients with ER$^-$ *p53*-mutated tumors, dose-intense cyclophosphamide was associated with a strikingly greater likelihood of a pCR (OR, 59.2; 95% CI, 6.4–544.4; $p < .0001$) (multivariate analysis in Table 3). Indeed, in patients with ER$^-$ *p53*-mutated tumors, the pCR rates were 71% ($n = 15$ of 21), 6% ($n = 1$ of 17), and 12% ($n = 7$ of 57) ($p < .0001$, χ^2 test) as the cyclophosphamide dose intensity was decreased (Fig. 2). Among the 21 ER$^-$ *p53*-mutated tumors treated by ddEC, 11 could be identified as typically triple negative and 9 of them reached pCR (81.2%). Eight overexpressed HER-2, among which 4 reached pCR. Data were unavailable for the 2 remaining patients, who both responded. The small number of ER$^+$ tumors and the low rate of pCR in this subset ($n = 6$ of 89 patients) precluded the reverse analysis. Although not randomized, these findings strongly suggest that dose-intense cyclophosphamide/anthracycline therapy is associated with greater efficacy in patients with ER$^-$ *p53*-mutated tumors, notably those with triple-negative tumors.

Table 3. Predictive parameters of pathologic complete response in patients with *p53*-mutated ER$^-$ breast cancer ($n = 95$), multivariate analysis

	Odds ratio (95% CI)	*p*-value
Age (<50 versus ≥50)	3.8 (0.9–15.3)	.06
Tumor size (cT0–cT2 versus cT3–cT4)	0.3 (0.04–2.8)	.3
Tumor grade (1 or 2 versus 3)	4.1 (0.5–32.8)	.18
Alkylating dose (high versus other)	59.2 (6.4–544.4)	.0001

Abbreviations: CI, confidence interval; ER, estrogen receptor.

Discussion

Use of high-dose alkylating agents in daily practice is a matter of very significant controversy. Two large, randomized trials in the adjuvant setting did not find a significant difference in outcome when increasing the dose of cyclophosphamide [28, 29]. Similarly, a randomized trial that evaluated high-dose alkylating agents with bone marrow transplantation did not report a significant effect on overall survival [30]. However, analysis of those trials was performed considering breast cancer as a single disease, which may have diluted out effects in specific subpopulations. Some previous molecular correlative studies have attempted to define which group could derive benefit from high-dose alkylating agents. In the National Surgical Adjuvant Breast and Bowel Project (NSABP) B25 trial, a subgroup of women aged <50 years or with ER-poor breast cancer (ER, 10–49 fmol/mg) seemed to derive a significant benefit from a higher dose of an alkylating agent [28]. Similarly, in a Dutch trial [31], p53 detection by IHC was associated with a significantly higher efficacy for high-dose

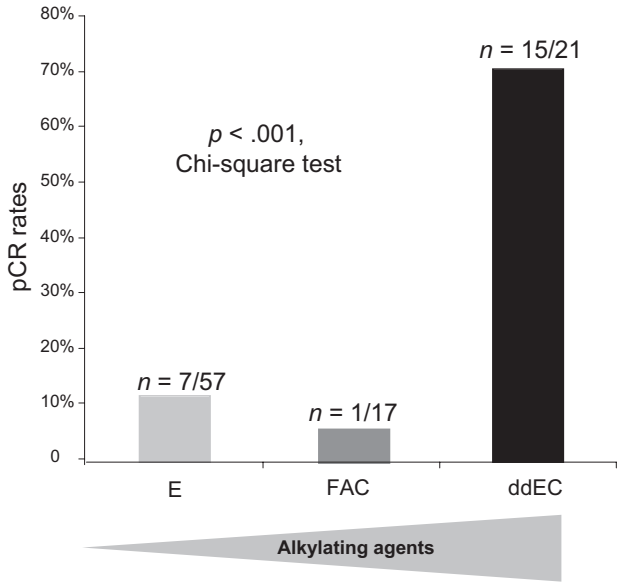

Figure 2. Efficacy of high-dose alkylating agents in patients with estrogen receptor–negative *p53*-mutated tumors.

Abbreviations: ddEC, dose-dense epirubicin and cyclophosphamide; E, single-agent epirubicin; FAC, 5-fluorouracil, doxorubicin, and cyclophosphamide; pCR, pathologic complete response.

cyclophosphamide, carboplatin, and thiotepa combination chemotherapy. Those two studies are fully in line with our findings. Although our study did not have complete annotations (HER-2, PR, and ER) available for all patients and lacked a central review for determination of pCR, it clearly identifies *p53*-mutant ER$^-$ tumors as those most sensitive to high-dose alkylating agents.

The technology used for p53 typing is critical, and the yeast functional assay used here is robust, assesses function (thus excluding passenger mutations), and is more sensitive than direct sequencing [10]. p53 inactivation is very unevenly distributed across the different molecular subclasses of breast cancer, ranging from none (luminal A) to virtually 100% (basal). This could suggest that p53 inactivation contributes to the basic biology of those tumors, as well as to their treatment response. p53 status critically regulates response to DNA-damaging agents, by promoting either cell-cycle arrest or apoptosis, with the opposite effect on tumor outcome [32, 33]. Our findings confirm the observations that dysfunctional p53 is predictive of chemoresistance to anthracyclines alone [34] and raise the tantalizing prospect that high-dose alkylating agents can actually reverse the intrinsic anthracycline resistance of ER$^-$ *p53*-mutated tumors, a proposal with considerable potential clinical importance.

Many *p53*-mutated and ER$^-$ breast cancers fall into the basal-like molecular subtype [10, 20], although some belong to the molecular apocrine one [26, 27] (data not shown). Future studies should determine the sensitivity of each molecular subclass to this combination. In addition to a defective p53 pathway, basal tumors may also have an impaired BRCA1 repair pathway [35], which may contribute to the high sensitivity of these tumors to alkylating agents. Several clinical trials showed that basal-like breast cancers are very sensitive to combination chemotherapy [36, 37]. Yet the rate of pCR achieved here with the dose-intense regimen in ER$^-$ *p53*-mutated tumors (70%) appears notably higher than in those previous trials. The rate of pCR even reached 80% in triple-negative tumors. Accordingly, in St. Louis Hospital, ER$^-$ *p53*-mutated tumors treated by neoadjuvant therapy are prescribed the ddEC regimen. In patients treated with the ddEC regimen, pCR was the only predictor of 10-year survival [38], suggesting that many of the complete remissions observed here will translate into actual cures. Our results identify p53 as a critical biomarker of high-dose cyclophosphamide sensitivity, in the context of anthracycline combinations. The magnitude of the observed effect and the size of the population under study make it most unlikely that the superior response of ER$^-$ *p53*-mutated tumors may be explained by patient heterogeneity. Thus, although this analysis carries several intrinsic limitations, our results warrant prospective randomized trials to directly test the hypothesis that dose-intense alkylating agents actually reverse anthracycline resistance in ER$^-$ *p53*-mutated breast cancers.

Acknowledgments

We thank all the technicians at the Biochemistry Department, Saint Louis Hospital (Evelyne Wittmer, Catherine Brunin, Dominique Chapelin, Martine Legrand, Claire Bocquet) for p53 status determination.

F.A. is supported by an ASCO career development award. L.P. is supported by the Breast Cancer Research Foundation. C.S. is supported by the Fonds National de la Recherche Scientifique. The TOP trial has received support from the Fondation Luxembourgeoise Contre le Cancer, the Brussels region, and the Fonds National de la Recherche Scientifique. Hd.T. is supported by Ligue Contre le Cancer, Région Ile de France, Programme Hospitalier de Recherche Clinique (PHRC), and INCa.

Author Contributions

Conception/design: Jacqueline Lehmann-Che, Fabrice André, Hugues de Thé
Provision of study materials or patients: Jacqueline Lehmann-Che, Fabrice André, Sylvie Giacchetti, Christine Desmedt, Marc Espié, Michel Marty, Philippe Bertheau, Christos Sotiriou, Martine Piccart, W. Fraser Symmans, Lajos Pusztai, Hugues de Thé
Collection/assembly of data: Jacqueline Lehmann-Che, Fabrice André, Christine Desmedt, Elisabeth Turpin, Louis-François Plassa, Philippe Bertheau, W. Fraser Symmans, Lajos Pusztai, Hugues de Thé
Data analysis and interpretation: Jacqueline Lehmann-Che, Fabrice André, Chafika Mazouni, Hugues de Thé
Manuscript writing: Jacqueline Lehmann-Che, Fabrice André, Chafika Mazouni, Lajos Pusztai, Hugues de Thé
Final approval of manuscript: Jacqueline Lehmann-Che, Fabrice André,

Christine Desmedt, Chafika Mazouni, Sylvie Giacchetti, Elisabeth Turpin, Marc Espié, Louis-François Plassa, Michel Marty, Philippe Bertheau, Christos Sotiriou, Martine Piccart, W. Fraser Symmans, Lajos Pusztai, Hugues de Thé

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