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Comprehensive Assessment of Genetic and Molecular Features Predicting Outcome in Patients With Chronic Lymphocytic Leukemia: Results From the US Intergroup Phase III Trial E2997

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ABSTRACT

Purpose
Genomic features including unmutated immunoglobulin variable region heavy chain (IgV_H) genes, del(11q22.3), del(17p13.1), and *p53* mutations have been reported to predict the clinical course and overall survival of patients with chronic lymphocytic leukemia (CLL). In addition, ZAP-70 and Bcl-2 family proteins have been explored as predictors of outcome.

Patients and Methods
We prospectively evaluated the prognostic significance of a comprehensive panel of laboratory factors on both response and progression-free survival (PFS) using samples and data from 235 patients enrolled onto a therapeutic trial. Patients received either fludarabine (FL; n = 113) or fludarabine plus cyclophosphamide (FC; n = 122) as part of a US Intergroup randomized trial for previously untreated CLL patients.

Results
Complete response (CR) rates were 24.6% for patients receiving FC and 5.3% for patients receiving FL ($P = .00004$). PFS was statistically significantly longer in patients receiving FC (median, 33.5 months for patients receiving FC and 19.9 months for patients receiving FL; $P < .0001$). The occurrence of del(17p13.1) (hazard ratio, 3.428; $P = .0002$) or del(11q22.3) (hazard ratio, 1.904; $P = .006$) was associated with reduced PFS. CR and overall response rates were not significantly different based on cytogenetics, IgV_H mutational status, CD38 expression, or *p53* mutational status. Expression of ZAP-70, Bcl-2, Bax, Mcl-1, XIAP, Caspase-3, and Traf-1 was not associated with either clinical response or PFS.

Conclusion
These results support the use of interphase cytogenetic analysis, but not IgV_H, CD38 expression, or ZAP-70 status, to predict outcome of FL-based chemotherapy. Patients with high-risk cytogenetic features should be considered for alternative therapies.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) has a widely variable clinical course. Thus, there is considerable interest in identifying molecular features of CLL that predict disease outcome and overall survival (OS) before initiation of therapy. Understanding the impact of genetic features on treatment outcome has the potential to allow CLL patients at high risk of early relapse, or reduced likelihood of response, to pursue alternative investigational therapies while avoiding unnecessary treatment-associated toxicities. Factors previously shown to be associated with a reduced time to initiation of treatment and poor survival include unmutated immunoglobulin variable region heavy chain (IgV_H) gene mutation status and associated ZAP-70 overexpression,[1-7] elevated CD38 expression,[6] select chromosomal abnormalities including del(17p13.1) and del(11q22.3),[8] and the presence of nonsilent *p53* mutations.[9] Irrespective of these, CLL generally is treated only at the onset of symptoms because of previous findings that early treatment with alkylator-based therapy does not influence OS.[10] For young and otherwise healthy patients, traditional therapy at development of symptomatic CLL includes fludarabine (FL), based on results of three randomized studies[11-13] that demonstrated significantly improved overall

response (OR), complete response (CR), and progression-free survival (PFS) with FL-based therapy relative to alkylator-based therapy. Subsequent trials demonstrated improved CR rates and PFS with the combination of fludarabine plus cyclophosphamide (FC).[14] However, these findings have yet to be confirmed in a prospective, randomized phase III trial.

We therefore conducted a randomized trial, Eastern Cooperative Oncology Group E2997/Cancer and Leukemia Group B 10103/Southwest Oncology Group E2997 (Intergroup trial E2997), to compare FL monotherapy versus the combination of FC. This trial enrolled symptomatic but previously untreated CLL patients who met National Cancer Institute (NCI) criteria[15] for initiation of therapy. Extensive correlative studies were included to examine the predictive value to clinical outcome of previously identified biomarkers. Although studies examining the usefulness of these assays have supplied important data, they largely have been retrospective analyses on nonrandomized clinical trials.[16-20] The primary objective of this report is to assess the impact of select molecular features on response and PFS in this patient population.

PATIENTS AND METHODS

Patients

Patients enrolled onto E2997 provided written informed consent to participate in the correlative studies. Eligibility criteria included symptomatic, untreated CLL as defined by NCI 1996 guidelines.[15] Treatment was randomly assigned centrally to either FL administered intravenously at 25 mg/m^2/d on days 1 through 5, repeated monthly for a maximum of six total treatments (FL alone), or FL administered intravenously at 20 mg/m^2/d on days 1 through 5 plus cyclophosphamide 600 mg/m^2 administered intravenously on day 1, repeated every 28 days for a maximum of six total treatments (FC), as described previously.[14]

Study End Points

Response assessments used NCI guidelines. PFS was defined from the date of random assignment to the date of progression or death (events) or last follow-up (censored), whichever came first, and estimated using the method of Kaplan and Meier. At the time of this analysis, OS data are immature. However, PFS survival data are mature based on the large number of reported clinical events.

Cell Isolation

Peripheral blood was obtained before treatment, shipped overnight at ambient temperature to our central processing laboratory, and mononuclear cells were isolated using Ficoll density-gradient centrifugation. Cells were then processed for lysate or RNA, or were cryopreserved. Quality-control studies were conducted to verify that overnight shipping did not influence the levels of proteins to be analyzed.

Interphase Cytogenetics

Probe sets designed to detect common anomalies associated with CLL by fluorescent in situ hybridization were described previously.[21] Two hundred cells were evaluated for each probe set, and results were expressed as the percentage of nuclei with an abnormal signal pattern for any probe set and corresponding chromosomal anomaly.

p53 Mutational Analysis

p53 exons 5 to 9 were amplified from genomic DNA and analyzed using denaturing gradient gel electrophoresis, as described.[22] Patient samples with identified *p53* mutations were then subjected to automated DNA sequencing for confirmation.

IgV$_H$ Mutational Analysis

IgV$_H$ gene mutational analysis was performed as described previously.[23,24] A small sample set was analyzed by both methods with no difference in results. Nucleotide sequences were aligned to best match in both IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/; National Cancer for Biotechnology Information, Bethesda, MD) and in the V Base sequencing directory (http://vbase.mrc-cpe.cam.ac.uk/).

Protein Analysis

Methods for analysis of Bcl-2, Bax, Mcl-1, Bag-1, XIAP, Caspase-3, and TRAF-1 were reported previously.[25] Lysate from the B-lymphoma cell line RS11846 was included on each blot as an arbitrary standard for subsequent normalization of all results. ZAP-70 was measured by immunoblot essentially as reported previously.[5] ZAP-70 expression was calculated relative to the housekeeping protein GAPDH, and the Jurkat T cell line was used as a positive control for normalization across immunoblots.

Flow Cytometry

Surface antigens were detected in whole blood specimens by three-color multiparameter flow cytometry as described previously.[26] Phycoerythrin (PE)-conjugated anti-CD38 antibody was used as recommended (Immunotech/Beckman Coulter, Fullerton, CA).

After they were fixed and permeabilized (Fix & Perm; Invitrogen, Grand Island, NY), isolated mononuclear cells were incubated with anti-ZAP-70 antibody clone 2F3.2 (Upstate/Millipore, Charlottesville, VA) followed by counterstaining with fluorescein-isothiocyanate–conjugated goat F(ab′)$_2$ antimouse immunoglobulin. CD3-PE-Cyanine 5 and CD19-PE (Immunotech) were added to detect simultaneously normal T cells or CLL cells, respectively. ZAP-70 staining of T lymphocytes was used as an internal control. Given that the number of contaminating T lymphocytes in most specimens was significantly lower than that of the CLL cells (median, 4%), at least 1,000 CD3-positive T cells were acquired. The intensity of ZAP-70 staining was expressed by mean fluorescence channel of anti-ZAP-70 divided by the mean fluorescence channel of the isotype control (mean fluorescence intensity or mean fluorescence intensity ratio). Subsequently, the ZAP-70 mean fluorescence intensity ratio in CLL cells divided by that in T cells for each specimen was calculated. In 226 of 251 patients, staining was performed on thawed cells; in 25 patients, fresh mononuclear cells were analyzed. The use of thawed versus fresh CLL cells yielded comparable results.

Statistical Analysis

All patients enrolled onto E2997 were eligible for the laboratory studies. The Wilcoxon rank sum test was used to investigate associations between categoric factors (such as dominant cytogenetic anomaly and mutation status) and continuous factors (such as time to treatment and protein expression levels). Associations between putative prognostic factors and response were assessed by the Fisher's exact test, and associations with PFS were assessed using the log-rank test. To control for the statistically significant treatment effect, logistic regression models including a treatment effect were used to investigate the relation of laboratory measures on achievement of CR or OR. Cox proportional hazards regression models including a treatment effect were used to assess the impact of laboratory measures on PFS. Associations between laboratory measures and response end points were also investigated within each treatment arm.

RESULTS

Patients and Treatment Outcome

A total of 278 patients were enrolled onto E2997, and 235 patients participated in the correlative studies. Patient characteristics of this subset are listed in Table 1. The CR rates were 5.3% for patients receiving FL alone and 24.6% for patients receiving FC ($P = .00004$). The PFS was statistically significantly improved in patients receiving FC versus FL alone (median PFS, 33.5 *v* 19.9 months, respectively; $P < .0001$). These results are consistent with those in E2997 as a whole. There was a significant effect of treatment arm on both CR and OR. The estimated odds ratio of achieving a CR associated with treatment arm (FC) was 5.82 (95% Wald confidence limits = 2.32, 14.59).

Table 1. Patient Characteristics

Treatment Assignment	FL (n = 113) No. of Patients	FL (n = 113) %	FC (n = 122) No. of Patients	FC (n = 122) %
Age, years				
Median	63		62	
Range	33-83		39-86	
Male	80	71	88	72
Rai stage				
0/1	33	29	37	30
2	34	30	30	25
3	23	20	24	20
4	23	20	31	25
Lymphadenopathy	98	87	107	88
Splenomegaly	73	65	71	58
IgV$_H$ mutation status				
IgV$_H$ unmutated (≥ 98%)	53	47	57	47
IgV$_H$ unmutated (≥ 97%)	63	56	68	56
Not available	19	17	21	17
Interphase cytogenetics				
del(17p)	9	8	10	8
del(11q)	16	14	24	20
del(6q)	1	1	7	6
+12	27	24	21	17
Normal	20	18	17	14
del(13q)	38	34	43	35
IgH	2	2	0	0
p53 mutation	14	12	8	7
Not available	5	4	8	7

Abbreviations: FL, fludarabine alone; FC, fludarabine plus cyclophosphamide; IgH, immunoglobulin heavy chain.

Clinical and Laboratory Correlative Data

Of the 235 patients with cytogenetics data who were assessable for clinical response, 195 (83%) had *IgV$_H$* mutational status known, 184 (78%) had ZAP-70 data by immunoblot analysis, 228 (97%) had *p53* mutational analysis, and 207 (88%) had quantitative data on apoptosis-related proteins. In a separate reference laboratory, 251 samples were analyzed for CD38 and ZAP-70 expression by flow cytometry.

IgV$_H$ *Gene Mutational Status*

Of the 195 samples with *IgV$_H$* mutational analysis, 110 samples (56.4%) were unmutated using the cutoff of 98% or greater identity with germline. Using a cutoff of 97% identity, 136 samples (69.7%) were unmutated.

Interphase Cytogenetic Abnormalities

Given that some abnormalities occur in combination, the Döhner hierarchical classification[8] for patients by treatment arm was used. The frequency of CR and OR did not vary significantly by cytogenetic group in the context of the model including treatment (data not shown). However, as listed in Table 2, PFS varied significantly across cytogenetic groupings within both the FC ($P = .04$) and FL alone ($P = .01$) treatment arms.

PFS data for the high-risk cytogenetic abnormalities del(17p) and del(11q) are shown in Figure 1 (PFS curves for other cytogenetics subsets are presented in Appendix Figs A1 and A2, online only). When the entire patient group was examined regardless of treatment, PFS was altered in patients with del(17p) alone, with a hazard ratio of 3.428 ($P = .0002$). The hazard ratio associated with a del(11q) alone was 1.904 ($P = .006$), indicating that the presence of either del(17p) or del(11q) is associated with shorter PFS. The overall median PFS was 10.8 months for patients with del(17p) and 21.5 months for those with del(11q). We next examined PFS of the del(17p) and del(11q) groups with respect to treatment arm. Although there was an indication of an improvement of PFS in patients with del(11q) on FC versus FL alone, this difference in duration was not statistically significant ($P = .19$). With longer follow-up, the *P* value associated with a treatment difference within del(11q) patients becomes .16; at 35 months, 18% of patients were progression free. Thus, there is no difference in this study between treatment arms for this group of patients with del(11q).

Previous studies have indicated the prognostic significance of *IgV$_H$* gene mutations.[1,2] Surprisingly, in untreated patients meeting treatment criteria, *IgV$_H$* gene mutational status did not predict a statistically significant difference in PFS (Fig 2; Appendix Fig A3, online only). The Kruskal-Wallis test assessing whether *IgV$_H$* mutated and

Table 2. Median PFS by Genomic Features

Feature	Total No. of Patients	PFS (months)	Patients Receiving FL	PFS for Patients Receiving FL (months)	Patients Receiving FC	PFS for Patients Receiving FC (months)
IgV$_H$ unmutated (≥ 98%)	110	20.8	53	15.0	57	31.4
IgV$_H$ mutated (< 98%)	85	29.6	41	23.4	44	NR
Interphase cytogenetics						
del(17p)	19	10.8	9	8.9	10	11.9
del(11q)	40	21.5	16	14.9	24	25.2
del(6q)	8	NR	1	26.7	7	NR
+12	48	23.4	27	21.4	21	NR
Normal	37	22.2	20	14.1	17	NR
del(13q)	81	27.6	38	22.9	43	NR
IgH	2	11.4	2	11.4	0	—
p53 mutation without del(17p)	15	33.3	10	36.7	5	33.3
No *p53* mutation without del(17p)	188	24.9	89	19.2	99	38.3

Abbreviations: PFS, progression-free survival; FL, fludarabine alone; FC, fludarabine plus cyclophosphamide; NR, median PFS not reached; IgH, immunoglobulin heavy chain.

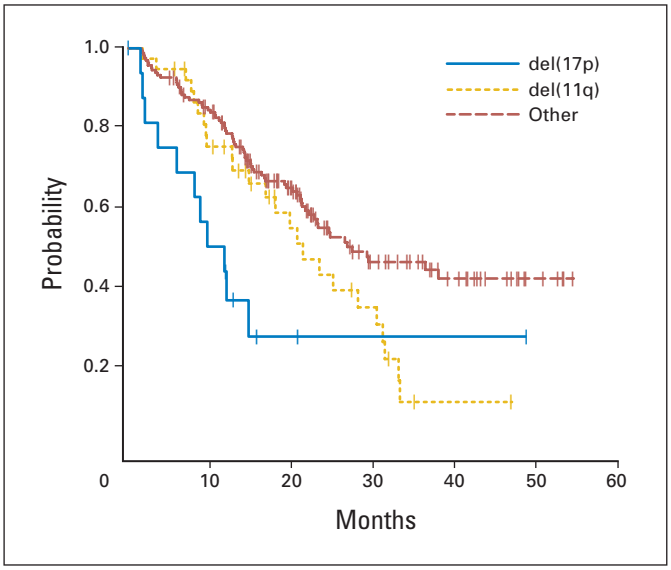

Fig 1. Progression-free survival for the high-risk cytogenetic abnormalities del(17p) and del(11q) in both treatment arms (n = 235). The *P* value for the comparison of del(17p), del(11q), and other cytogenetic anomalies is .0006 by the log-rank test.

unmutated patients followed the same distribution across cytogenetics categories yielded a *P* value of .0001, suggesting that there is an association between cytogenetics category and IgV_H gene mutational status (Appendix Tables A1 and A2, online only).

p53 *Mutations*

Of the 228 patients examined for *p53* mutations in exons 5 to 9, 28 mutations were identified in 25 patients (11%). Of the 22 patients that were also studied by fluorescent in situ hybridization, seven exhibited a del(17p). The impact of *p53* mutational status on attaining an objective remission did not reach statistical significance in either arm. In patients with a mutation of *p53* but without del(17p), there

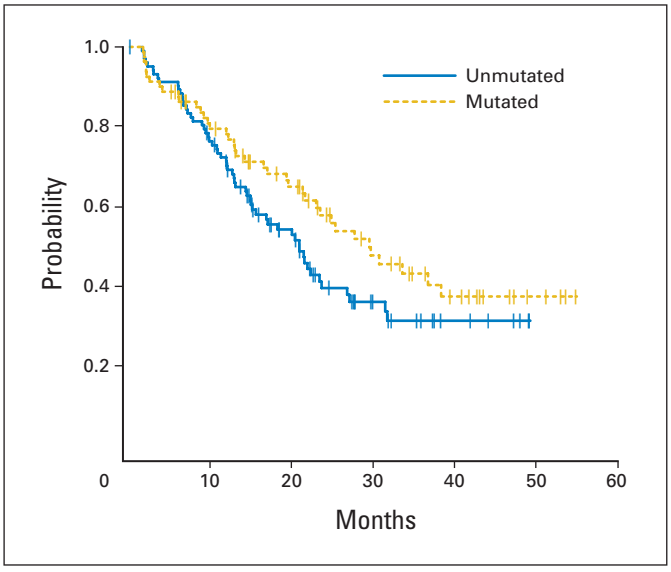

Fig 2. Progression-free survival relative to IgV_H gene mutational status in both treatment arms (n = 195). The *P* value for this comparison, using the 98% cutoff, is .08 by the log-rank test.

was no enhancement of the model predicting poorer outcome (hazard ratio, 1.082; $P = .809$). Therefore, detailed identification of *p53* mutations did not contribute to establishing the prognosis for clinical outcome in this population (ie, predicting either achievement of a response or response duration). Specific details of the *p53* mutations identified are provided in Appendix Table A4 (online only).

ZAP-70 Determinations and CD38 Expression

ZAP-70 protein was quantified by immunoblot analysis in peripheral-blood mononuclear cells. Even when analysis was restricted to samples with fewer than 10% CD3-positive cells (n = 159), this parameter did not predict the likelihood of achieving a response or the time to progression of disease. Furthermore, flow cytometric analysis of ZAP-70 together with CD3 and CD19 determinations in samples of peripheral blood from these patients also failed to predict for response or duration of remission.

High CD38 expression in leukemic cells from CLL patients has been reported to correlate with aggressive disease. We explored a range of values from 7% to 35% of cells with CD38-positive status. These various measures of CD38 expression performed equally poorly in identifying patients who would achieve CR, using logistic regression models that included randomized treatment arm. When a continuous value of CD38 expression is used, the *P* value in a model including study treatment arm is .53. The *P* value is .52 if a high value of CD38 expression at 30% is used, and $P = .54$ if a high value of either 35% or 7% is used. Therefore, in this study CD38 expression did not predict response to a FL-based therapy.

Quantitative Apoptosis-Related Protein Analysis

The expression of apoptosis-related proteins was assessed on 207 baseline samples (FC, n = 114; FL alone, n = 103; data are provided in Appendix Table A3, online only). The response rates for patients in this subset were quite similar to the overall rates observed in E2997 as a whole (data not shown). The Wilcoxon rank sum test was used to explore whether any of these proteins were associated with achievement of a CR, given that this was a primary end point of the clinical study. Of the proteins examined, none achieved a statistically significant association with either complete remission or PFS. In considering OR (CR plus partial response [PR]), a lower Mcl-1/Bax protein ratio is associated with OR on this arm ($P = .02$). This finding is in agreement with earlier work by members of our group.[25]

Models Combining Clinical and Laboratory Predictors of Outcome

The model for achievement of CR that included treatment (FL alone *v* FC) could not be improved by inclusion of laboratory parameters. The treatment arm (FC) carried a hazard ratio of 0.417 ($P < .0001$), and was the only protective variable in the model. However, cytogenetic analysis substantially impacted the modeling of PFS. Occurrence of del(17p) was associated with a hazard ratio of 3.428 ($P = .0002$), and del(11q) was associated with a hazard ratio of 1.904 ($P = .0063$). The remaining laboratory parameters examined (apoptosis-related proteins, ZAP-70, *p53* mutations, CD38 expression, and IgV_H status) did not predict achievement or duration of response.

DISCUSSION

We describe the first prospective study in CLL patients of molecular markers previously demonstrated to be associated with survival, and

examine their prognostic significance relative to treatment outcome with FL-based chemotherapy. Our results are confined to a population of patients who were untreated but required treatment based on NCI criteria. In this group, we found that the high-risk cytogenetic abnormalities del(17p13.1) and del(11q22.3) significantly correlate with a shortened PFS when either FL alone or FC is administered. Furthermore, in a multivariate analysis that included treatment assignment, these two features were identified as being significant risk factors for early relapse. The significant overlap of del(11q) and unmutated IgV_H gene status prevented inclusion of this latter molecular marker into the multivariate analysis. This strong association of high-risk cytogenetic abnormalities with IgV_H unmutated status confirms earlier findings.[27,28] To date, no study in CLL has prospectively examined the impact of these abnormalities together, nor their clinical relevance after receipt of effective standard therapy. Furthermore, this is the first study to show that *p53* mutations in the absence of del(17p) do not have an independent negative impact on PFS. An earlier study examining *p53* mutations, deletions, and promoter methylation showed that these alterations do not predict individual response to FL in patients with advanced CLL.[29] Although this was a retrospective study using a reduced sample size, the conclusions are in agreement with our data.

We also assessed the influence of ZAP-70 and CD38 expression as well as specific apoptosis-related proteins on clinical outcome, but detected no significant correlation of these parameters with response to therapy. It is important to note that protein studies were performed using peripheral-blood mononuclear cells, and T cells may have confounded accurate assessment of ZAP-70 in some samples. However, samples typically had significantly elevated percentages of CD19-positive cells; furthermore, our analysis is similar to that performed by Crespo et al.[5] In contrast to what has been reported, ZAP-70 expression was not associated with IgV_H gene mutational status.

This study enhances our understanding of appropriate risk stratification by demonstrating that patients with either del(17p) or del(11q) have a significantly inferior PFS after initial response to therapy with either FL alone or FC. This finding supports the assertion that alternative treatments should be pursued for this patient population. A surprising observation was that these genetic features failed to identify patients who would not respond to initial therapy. In contrast, deletions of *p53* previously have been associated with poor response to fludarabine and rituximab.[9,16] Reasons for this discordance between our study and work from other groups might include application of different response criteria and a smaller number of patients in the earlier analyses.

Given the overall clinical results of E2997, the therapeutic approach of FC will likely be part of the backbone of future phase III studies in CLL. Despite earlier predictions of high complete remission rates with FL-based combinations, other large well-controlled clinical trials have also reported similar responses both to FL alone and FC.[30] Our findings are relevant because they demonstrate the existence of a high-risk patient subset that would likely benefit from alternative novel therapies.

It is of interest that these same prognostic factors also adversely influence outcome among patients receiving FL and rituximab, as recently reported by the Cancer and Leukemia Group B.[18] Application of the results of these studies might include the design of risk-adapted therapy for CLL, similar to that currently performed in patients with acute leukemia. In this scenario, well-tolerated therapies such as FC or FL plus rituximab are used in low-risk patients, whereas more intensive or investigational treatments are applied in patient groups with high-risk cytogenetic features. More aggressive therapies could include alemtuzumab[31] or newer therapeutic agents such as flavopiridol that have significant activity in refractory patients or patients with poor-risk cytogenetic features.[32] Early application of nonmyeloablative allogeneic stem-cell transplantation might also be considered as part of future clinical trials in high-risk patients, due to the short remission observed with any standard therapy and presumed diminished efficacy after relapse.[33,34] It is anticipated that the use of innovative therapeutic strategies may ultimately improve the outcome of patients predicted to do poorly with standard chemotherapy.

The use of ZAP-70 protein expression, interphase cytogenetics, and IgV_H mutational status can identify those patients at diagnosis who are more likely to experience disease progression, but does not identify those likely to respond to initial chemotherapy. The use of appropriate biomarkers [ie, del(17p) and del(11q)] before treatment to identify patients at increased risk for poor or short-term response will facilitate efforts to improve their ultimate outcome. It has been recommended that new diagnostic tools be systematically examined in the context of therapeutic trials for CLL patients.[35] Our data support the design of future trials based on risk-adapted therapeutic strategies; it is hoped that these strategies will improve outcome for those patients predicted to have a shorter duration of response.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).