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ORIGINAL ARTICLE

Prognostic and predictive value of the Immunoscore in stage III colon cancer patients treated with oxaliplatin in the prospective IDEA France PRODIGE-GERCOR cohort study†

F. Pagès[1,2,3,4*], T. André[5], J. Taieb[2,6], D. Vernerey[7,8], J. Henriques[7,8], C. Borg[9], F. Marliot[1,2,3,4], R. Ben Jannet[10,11], C. Louvet[12], L. Mineur[13], J. Bennouna[14], J. Desrame[15], R. Faroux[16], A. Kirilovsky[2,3,4], A. Duval[17], P. Laurent-Puig[2,18], M. Svrcek[5,17], F. Hermitte[19], A. Catteau[19], J. Galon[2,3,4‡] & J.-F. Emile[9,10,11‡]

[1]Department of Immunology, Européen Georges Pompidou Hospital, AP-HP, Paris; [2]French National Institute of Health and Medical Research (INSERM), Centre de Recherche des Cordeliers, Sorbonne Université, Sorbonne Paris Cité, Université Paris Descartes, Université de Paris, Paris; [3]Laboratory of Integrative Cancer Immunology, INSERM, Centre de Recherche des Cordeliers, Paris; [4]Equipe Labellisée Ligue Contre le Cancer, Paris; [5]Department of Medical Oncology, Saint-Antoine Hospital, Sorbonne University and AP-HP, Paris; [6]Department of Gastroenterology and Digestive Oncology, Européen Georges Pompidou Hospital, AP-HP, Paris; [7]Methodology and Quality of Life in Oncology Unit, Besançon University Hospital, Besançon; [8]University Bourgogne Franche-Comté, INSERM, EFS BFC, UMR1098, Interactions Hôte-Greffon-Tumeur/Ingénierie Cellulaire et Génique, Besançon; [9]University Hospital (CHRU) Besançon, UMR1098 UBFC/EFS/INSERM, Immuno-Oncologie et Biotechnologies en Cancérologie, Besançon; [10]EA4340-Biomarqueurs et essais cliniques en Cancérologie et Onco-Hématologie (BECCOH), Versailles University, Boulogne; [11]Ambroise Paré Hospital, AP-HP, Boulogne; [12]Department of Medical Oncology, Institut Mutualiste Montsouris, Paris; [13]Department of Radiation Therapy, Institut Sainte Catherine, Avignon; [14]Department of Medical Oncology, University Hospital of Nantes, Nantes; [15]Department of Medical Oncology, Private Hospital Jean Mermoz, Lyon; [16]Department of Gastroenterology, CHD Vendée, La Roche sur Yon; [17]Sorbonne University and INSERM, UMRS 938, Équipe Instabilité des microsatellites et cancer, Saint Antoine Research Center, Equipe Labellisée Ligue Contre le Cancer and SIRIC CURAMUS, AP-HP, Paris; [18]Department of Biology, Européen Georges Pompidou Hospital, AP-HP, Paris; [19]HalioDx, Marseille, France

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Background: The Immunoscore (IS), which prognostically classifies stage I–III colon cancer (CC) patients, was evaluated in the International Duration Evaluation of Adjuvant Therapy (IDEA) France cohort study investigating 3 versus 6 months of oxaliplatin-based adjuvant chemotherapy in stage III CC patients.

Patients and methods: Densities of CD3+ and CD8+ T cells in the tumor and invasive margin were determined by immunohistochemistry, quantified by digital pathology, and converted to IS. Mismatch repair status was determined by immunohistochemistry or by pentaplex PCR. Prediction of disease-free survival (DFS) by IS was analyzed by a multivariable Cox regression model in each study arm. Harrell's C-statistics were used to investigate the IS performance.

Results: Samples of 1322 patients were available. IS Low, Intermediate (Int), and High were observed in 43.6%, 47.0%, and 9.4% of patients, respectively. IS Low identified patients at higher risk of relapse or death compared with Int + High [hazard ratio (HR) = 1.54; 95% confidence interval (CI) 1.24–1.93, $P = 0.0001$]. The 3-year DFS was 66.80% (95% CI 62.23–70.94) for IS Low and 77.14% (95% CI 73.50–80.35) for IS Int + High. In multivariable analysis, IS remained significantly independently associated with DFS ($P = 0.003$) when adjusted for sex, histological grade, T/N stage, and microsatellite instability. For mFOLFOX6-treated patients (91.6% of the cohort), a statistical significant interaction was observed for the predictive value of IS for treatment duration (3 versus 6 months) in terms of DFS ($P = 0.057$). IS Int + High significantly predicted benefit of 6 months of treatment (HR = 0.53; 95% CI 0.37–0.75; $P = 0.0004$), including clinically low- and high-risk stage III CC (all $P < 0.001$). Conversely, patients with IS Low (46.4%) did not significantly benefit from the 6-month mFOLFOX6 versus the 3-month mFOLFOX6.

Conclusions: The prognostic value of IS for DFS was confirmed in patients with stage III CC treated with oxaliplatin-based chemotherapy. Its predictive value for DFS benefit of longer duration of mFOLFOX6 adjuvant treatment was found in IS Int + High. These results will be validated in an external independent cohort.

ClinicalTrials.gov registration: NCT03422601; EudraCT Number: 2009-010384-16.

Key words: biomarker, chemotherapy, colon cancer, Immunoscore, predictive, prognostic

*Correspondence to: Prof. Franck Pagès, Department of Immunology, Immunomonitoring Platform, Laboratory of Immunology, Hôpital Européen Georges Pompidou, 20 Rue Leblanc, 75015 Paris, France. Tel: +33 1 56 09 39 46
E-mail: franck.pages@aphp.fr (F. Pagès).

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‡Equal contribution.

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INTRODUCTION

Six months of adjuvant chemotherapy with fluoropyrimidines and oxaliplatin is the standard of care in patients with stage III colon cancer (CC).[1,2] The ability to reduce the treatment duration (3 versus 6 months) of 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) or capecitabine and oxaliplatin (CAPOX) without efficacy loss was investigated in the International Duration Evaluation of Adjuvant Therapy (IDEA) study.[3] Noninferiority of 3 versus 6 months was not shown. Among patients treated with FOLFOX, 6 months of adjuvant therapy was superior to 3 months in terms of disease-free survival [DFS; hazard ratio (HR) = 1.16, 95% confidence interval (CI) 1.06−1.26, superiority $P = 0.001$]. However, the prespecified noninferiority criterion of 3 versus 6 months was met in the CAPOX-treated patients.[3] These results were consistent with the IDEA France study, in which the majority of patients (∼90%) were treated with the mFOLFOX6 regimen.[4]

Biomarkers would be very helpful for guiding the choice and duration of adjuvant chemotherapy. Microsatellite instability (MSI) is currently the only biomarker for clinical use, but it is informative for only ∼10% of stage III CC patients.[5] Interestingly, a good prognostic value of MSI has been related to a high level of tumor-infiltrating lymphocytes,[6] which indicates a favorable prognosis in numerous solid tumors.[7] In colorectal cancer, we demonstrated that clinical outcome indicators strongly correlate with the quality and intensity of *in situ* adaptive immune reaction.[8−10] A standardized immune-based consensus assay termed Immunoscore (IS; i.e. the combination of total CD3+ and cytotoxic CD8+ T-cell densities in the tumor and its invasive margin) has been developed.[11] Its robustness and prognostic performance have been consolidated through an international validation study of patients with stage I−III CC.[12] The primary objective of this prospective ancillary study was to validate the prognostic performance of the IS test in the IDEA France cohort study. The predictive value of IS for DFS benefit of longer duration of adjuvant treatment was further investigated.

PATIENTS AND METHODS

Design and participants

IDEA France[4] was a multicenter, two-arm, open-label, randomized phase III trial with an accrual goal of 2000 stage III CC patients within the IDEA international collaboration ($N = 12834$)[3] comparing 3 versus 6 months of adjuvant fluoropyrimidine and oxaliplatin-based chemotherapy after surgery (EudraCT number, 2009-010384-16; For details, see supplementary Methods, available at *Annals of Oncology* online). The study was done in accordance with the declaration of Helsinki and Good Clinical Practice guidelines. All patients provided written informed consent. Approval of the protocol was obtained from an independent ethics committee. This prospective ancillary biomarker analysis was designed to validate the prognostic and predictive value of IS (ClinicalTrials.gov ID NCT03422601). The flowchart of the current analysis is shown in supplementary Figure S1, available at *Annals of Oncology* online.

Tumor sampling and Immunoscore testing

For each patient, one formalin-fixed paraffin-embedded tumor sample was collected for histology review (Ambroise Paré Hospital, Boulogne, France). Samples were sent to two laboratories (Immunomonitoring Platform, Européen Georges Pompidou Hospital, AP-HP, INSERM, Paris; HalioDx, Marseille, France) where an IS testing was carried out blinded to clinical data. In brief, sections of formalin-fixed paraffin-embedded tissues were incubated with rabbit monoclonal antihuman CD3+ (clone HDx2; HalioDx) and mouse monoclonal antihuman CD8+ (clone HDx1; HalioDx). Counterstained slides were digitalized at ×10 magnification and 0.45 µm/pixel resolution (NanoZoomer-XR, Hamamatsu, Japan) and CD3+ and CD8+ stained cells were quantified with the Image analysis software (Immunoscore Analyzer; HalioDx). Detailed protocol is provided in supplementary Methods, available at *Annals of Oncology* online. CD3+ and CD8+ T-cell densities were converted into IS with predefined cut-offs[12] as illustrated in Figure 1.

Tumor sampling and mismatch repair testing

Mismatch repair (MMR) tumor status was determined by immunohistochemistry carried out on tissue microarrays. Tumors were classified as MMR proficient when nuclear positivity was detected on tumor cells with both PMS2 and MSH6 primary antibodies and as MMR deficient when all tumor cells were negative with either PMS2 or MSH6, while positive nontumor cells were present in the same sample. When immunohistochemistry was not conclusive, a tumor MMR status was determined using pentaplex PCR.

Statistical analysis

DFS was estimated using the Kaplan−Meier method, described using median or rate at specific time points with 95% CI, and compared with the log-rank test. Follow-up duration was calculated using a reverse Kaplan−Meier estimation when feasible. The Cox proportional hazard models were used to estimate HR and 95% CI for factors associated with DFS. The association of baseline parameters with DFS was first assessed using a univariate Cox analysis and then parameters with P values <0.05 were entered into a final multivariable Cox regression model, after considering collinearity among variables using a correlation matrix. The assumption of proportionality was checked by plotting log-minus-log survival curves and by cumulative martingale process plots. Subgroup analysis for the IS and DFS association was carried out and summarized with forest plots. An interaction term within each subgroup was included in the regression Cox model between IS and its interaction. An interaction was significant if $P < 0.1$. All analyses were carried out using SAS version 9.4 (SAS Institute, Cary, NC) and R software version 2.15.2 (R Development Core Team, Vienna, Austria; http://www.r-project.org). All tests were

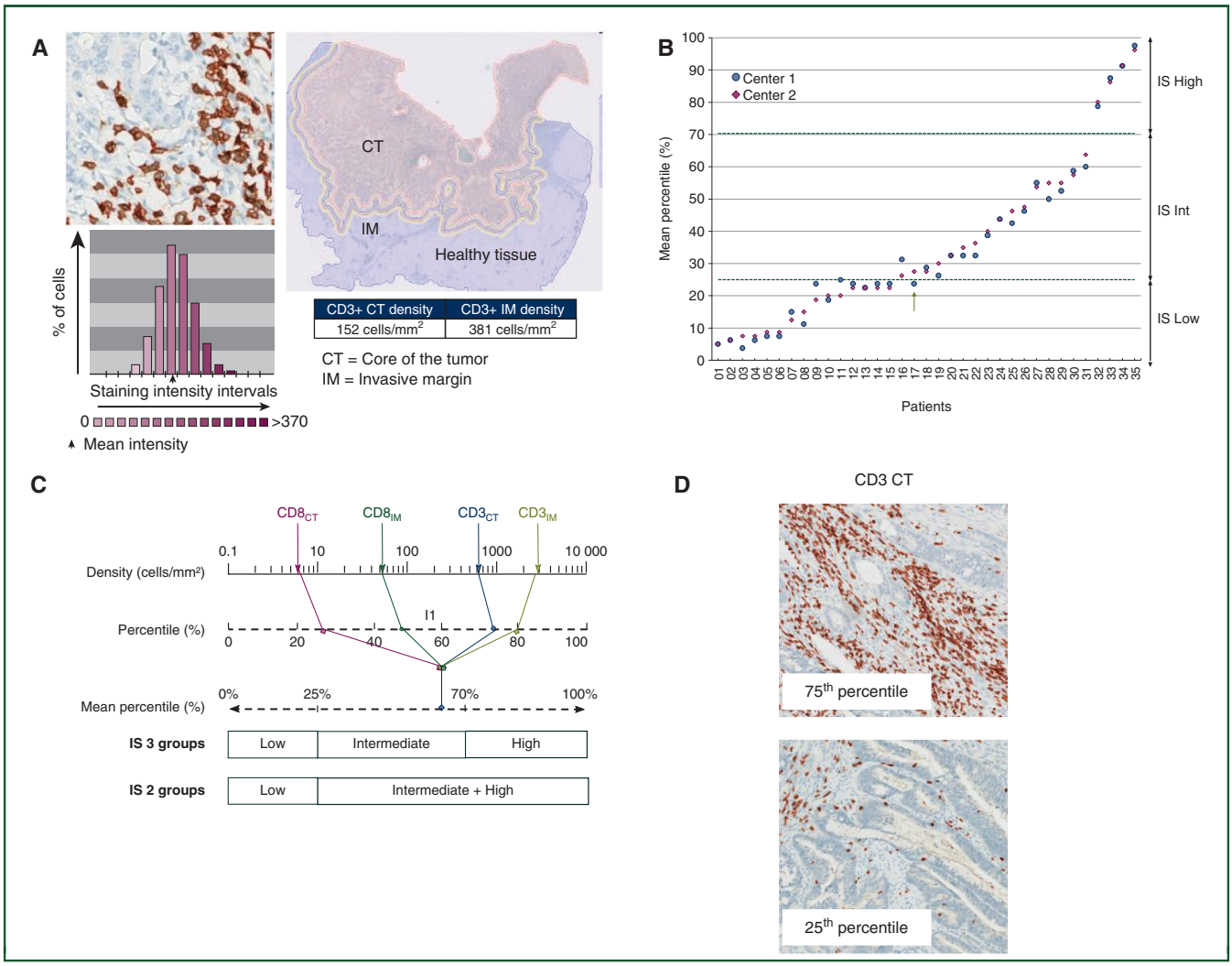


Figure 1. Determination of Immunoscore (IS) by image analysis software.
(A) Top left: illustration of the CD3+ T-cell detection, surrounded in red (magnification ×200). Bottom left: a histogram of the CD3+ T-cell staining intensities. Right: Automatic detection of the tumor (red), invasive margin (yellow), and healthy tissue (blue). (B) Reproducibility of the mean percentile (CD3+ and CD8+) for adjacent slides stained for CD3+ and CD8+ in 35 control cases between two assessment centers. The arrow shows the single discrepancy in IS translation between the two centers. (C) Chart illustrating the IS calculation method. Densities of CD3+ and CD8+ in the tumor and invasive margin converted into percentile values. The mean of the four percentiles obtained calculated and translated into IS. (D) An example of the CD3+ T-cell densities at 75th and 25th percentile in selected colon tumor.

two-sided (details in supplementary Methods, available at *Annals of Oncology* online).

RESULTS

Patient characteristics and IS determination

A total of 1322 (65.8%) patients of the overall IDEA France modified intention to treat (mITT) population with available samples were included in the current analysis (supplementary Figure S1, available at *Annals of Oncology* online). A lower proportion of clinically low-risk patients (UICC TNM 8th edition; T1—T3/N1), especially those with T1 tumors, was observed in the population with available samples as compared with the IDEA France mITT population (supplementary Table S1, available at *Annals of Oncology* online). Dedicated software monitored the CD3+ and CD8+ staining intensities (Figure 1A), allowing for consistency of stained cell counts[12] and the IS reproducibility (>97%) between the two assessment centers (Figure 1B), with only one discrepancy observed for a patient whose IS was very close to the cut-off point between IS Low and IS Int. Overall, 1062 (85.6%) cases reached the quality control. A total of 463 (43.6%) patients were IS Low, 499 (47%) were IS Int, and 100 (9.4%) were IS High (Figure 1C and D). IS categories were significantly correlated with T stage, T/N stage (T1—3 and N1 versus T4 and/or N2), and MSI (supplementary Table S2, available at *Annals of Oncology* online).

Association of Immunoscore with DFS

IS identified two populations (IS Low and IS Int + High) with significantly different survival profiles for DFS in univariate analysis (IS Low versus IS Int + High; HR = 1.54; 95% CI 1.24—1.93; P = 0.0001; Figure 2A and supplementary Table S3, available at *Annals of Oncology* online). The 3-year DFS was 66.80% (95% CI 62.23—70.94) and 77.14% (95% CI 73.50—80.35) for IS Low and IS Int + High, respectively. In addition, IS stratified into three categories

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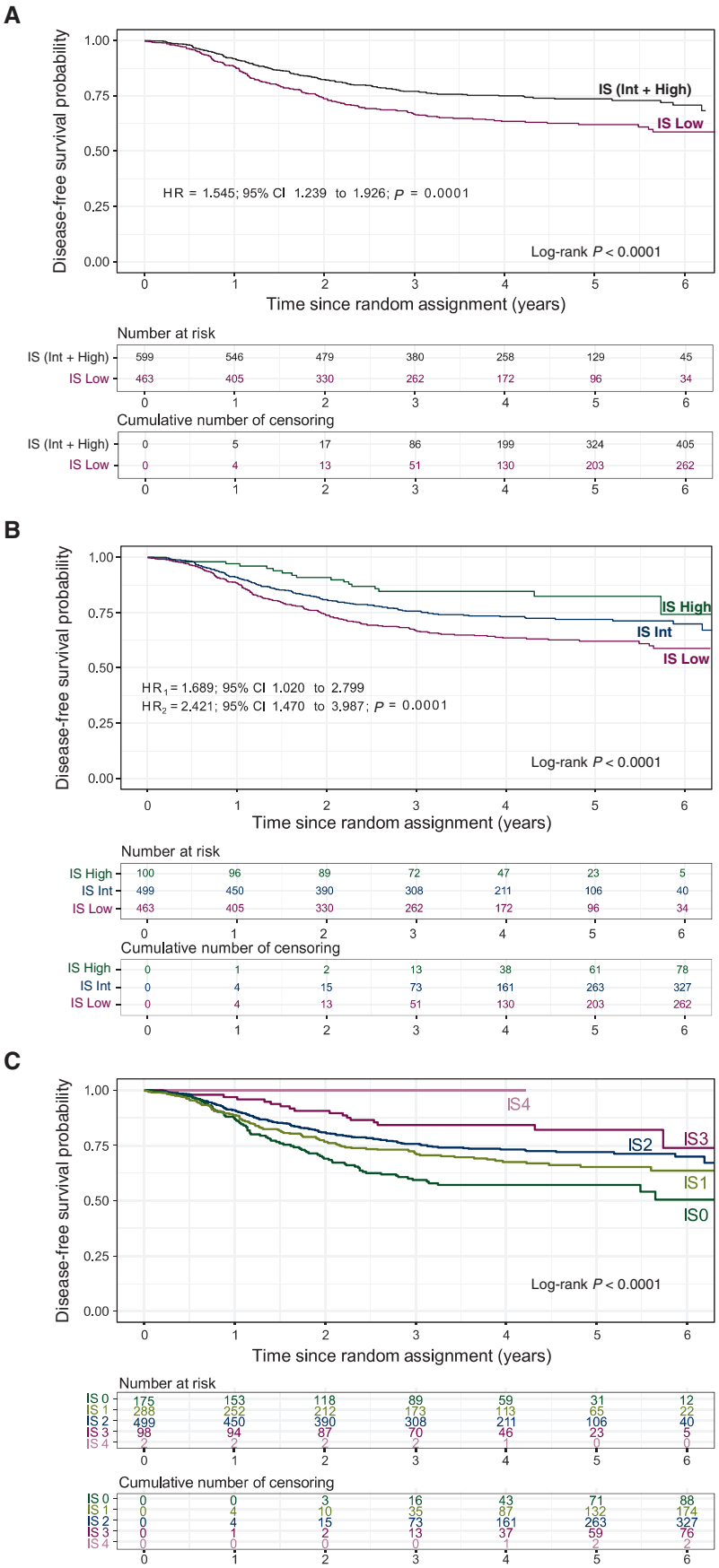


Figure 2. Kaplan–Meier curves of disease-free survival (DFS) according to Immunoscore (IS) in the overall modified intention-to-treat population.
(A) Regrouping IS into two categories [IS Low (burgundy), IS Intermediate (Int) + High (black)], (B) into three categories [IS Low (burgundy), IS Int (blue), and IS High (green)], and (C) into five categories (prespecified mean percentile; 0–10; >10–25; >25–70, >70–95, >95–100). CI, confidence interval; HR, hazard ratio.

(Low, Int, and High; Figure 2B), five categories (Figure 2C), and as a continuous variable (i.e. IS mean %; see supplementary Table S3, available at *Annals of Oncology* online) further discriminated patient outcomes for DFS (log-rank tests, all $P < 0.0001$). The 3-year DFS was 84.7% in patients with IS High versus 66.8% for those with IS Low.

Sex, T stage, N stage, T/N stage, histological grade, and MSI were also significantly associated with DFS, whereas Eastern Cooperative Oncology Group (ECOG) performance status and perforation were close to statistical significance (supplementary Table S3, available at *Annals of Oncology* online). In multivariable analysis, IS grouped into two categories (Low, Int + High; data not shown) or into three categories (Low, Int, and High) was significantly and independently associated with DFS (HR IS Low versus IS High = 2.22; 95% CI 1.31–3.77, $P = 0.003$) when combined with sex, histological grade, T stage, N stage, and MSI (Table 1). IS remained also independently associated with DFS (HR IS Low versus High = 2.28; 95% CI 1.39–3.76, $P = 0.001$) when combined with T/N stage (high-risk T4 and/or N2 versus low-risk T1–3, N1). The addition of IS to T/N stage significantly improved the model discrimination capacity (bootstrap C index mean difference = 0.022; 95% CI 0.005–0.04).

Table 1. Multivariate analyses of DFS according to three IS categories (Low, Intermediate, and High)

	Multivariate analysis ($N = 969$, N events = 284)				
	No.	Events	HR	95% CI	*P*
IS					
High	92	16	1		
Intermediate	465	123	1.685	0.99–2.87	
Low	412	145	2.224	1.31–3.77	0.003
Sex					
Female	408	103	1		
Male	561	181	1.307	1.02–1.67	0.031
Tumor stage					
T1–3	775	206	1		
T4	194	78	1.634	1.26–2.12	0.0003
Node stage					
N1 (1–3)	712	162	1		
N2 (≥4)	257	122	2.308	1.82–2.97	<0.0001
Histological grade					
Well or moderately differentiated	898	258	1		
Slightly or not differentiated	71	26	0.696	0.46–1.05	0.0871
MMR status					
pMMR	899	274	1		
dMMR	70	10	0.525	0.27–1.00	0.050
	Multivariate analysis ($N = 1060$, N events = 317)				
IS					
High	99	17	1		
Intermediate	499	133	1.717	1.04–2.84	
Low	462	167	2.283	1.39–3.76	0.001
Histopathological classification					
T1–2–3 and N1	622	129	1		
T4 and/or N2	438	188	2.347	1.87–2.94	<0.0001

CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; IS, Immunoscore; MMR, mismatch repair; pMMR, proficient mismatch repair; dMMR, deficient mismatch repair.

Subgroup analysis

Forest plots summarizing HR of DFS according to IS into two (Low versus Int + High) or three categories (Low, Int, and High) in prespecified subgroups are provided in supplementary Figures S2 and S3, available at *Annals of Oncology* online. A significant interaction term was identified with T stage ($P = 0.021$). A correlation was observed between the IS and occurrence of relapse or death in the subgroup of patients with clinically low-risk T1–3 and N1 tumor (log-rank tests, all $P \leq 0.001$; Figure 3A and supplementary Figure S4, available at *Annals of Oncology* online). In patients with high-risk T4 and/or N2 tumors, the IS categorization did not reach significance, but the lowest risk of event was observed with the highest IS (Figure 3A) and the risk of recurrence or death gradually increased along with decreasing IS (Figure 3B).

Predictive value of Immunoscore for duration of adjuvant mFOLFOX6 chemotherapy

A total of 492 and 481 patients treated with mFOLFOX6 and having available IS in the 3- and 6-month arms, respectively, were available for analysis. The group of patients receiving CAPOX (89/1062; 8.4% of the study cohort) was removed from analysis due to the small size giving low power to detect benefit. Clinical and histopathological characteristics and the IS categorizations were well balanced among patients receiving 3 and 6 months of mFOLFOX6 (supplementary Table S4, available at *Annals of Oncology* online). A significant interaction for the predictive value of IS for treatment duration (3 versus 6 months) in terms of DFS was observed for the whole mFOLFOX6 population ($P = 0.057$, supplementary Figure S5, available at *Annals of Oncology* online). A beneficial effect of the 6-month mFOLFOX6 regimen in terms of DFS was observed in patients with IS Int + High as compared with the 3-month mFOLFOX6 regimen (HR = 0.528; 95% CI 0.372–0.750; $P = 0.0004$, Figure 4A and supplementary Table S5, available at *Annals of Oncology* online). The benefit of 6-month mFOLFOX6 therapy in patients with IS Int + High was retained in those with clinically high-risk tumors (T4 and/or N2 tumors; HR = 0.54, $P = 0.007$, Figure 4B) and with clinically low-risk tumors (T1–T3 and N1; HR = 0.47, $P = 0.010$, Figure 4C). Indeed, in IS Int + High patients with clinically low-risk tumors, the 3-year DFS was 80.8% (95% CI 73.95–86.05) in those receiving 3 months of mFOLFOX6 treatment as compared with 91.4% (95% CI 85.8-94.8) in the 6-month group. Unlike IS Int + High patients, patients with IS Low did not experience significant benefit from the 6-month mFOLFOX6 regimen as compared with the 3-month regimen (HR = 0.84, $P = 0.269$, Figure 4A and supplementary Table S5, available at *Annals of Oncology* online). This observation was retained in clinically low- and high-risk tumors (Figure 4B and C and supplementary Table S5, available at *Annals of Oncology* online).

DISCUSSION

The IS in two (primary objective) and three categories was validated as a prognostic factor for DFS in stage III CC

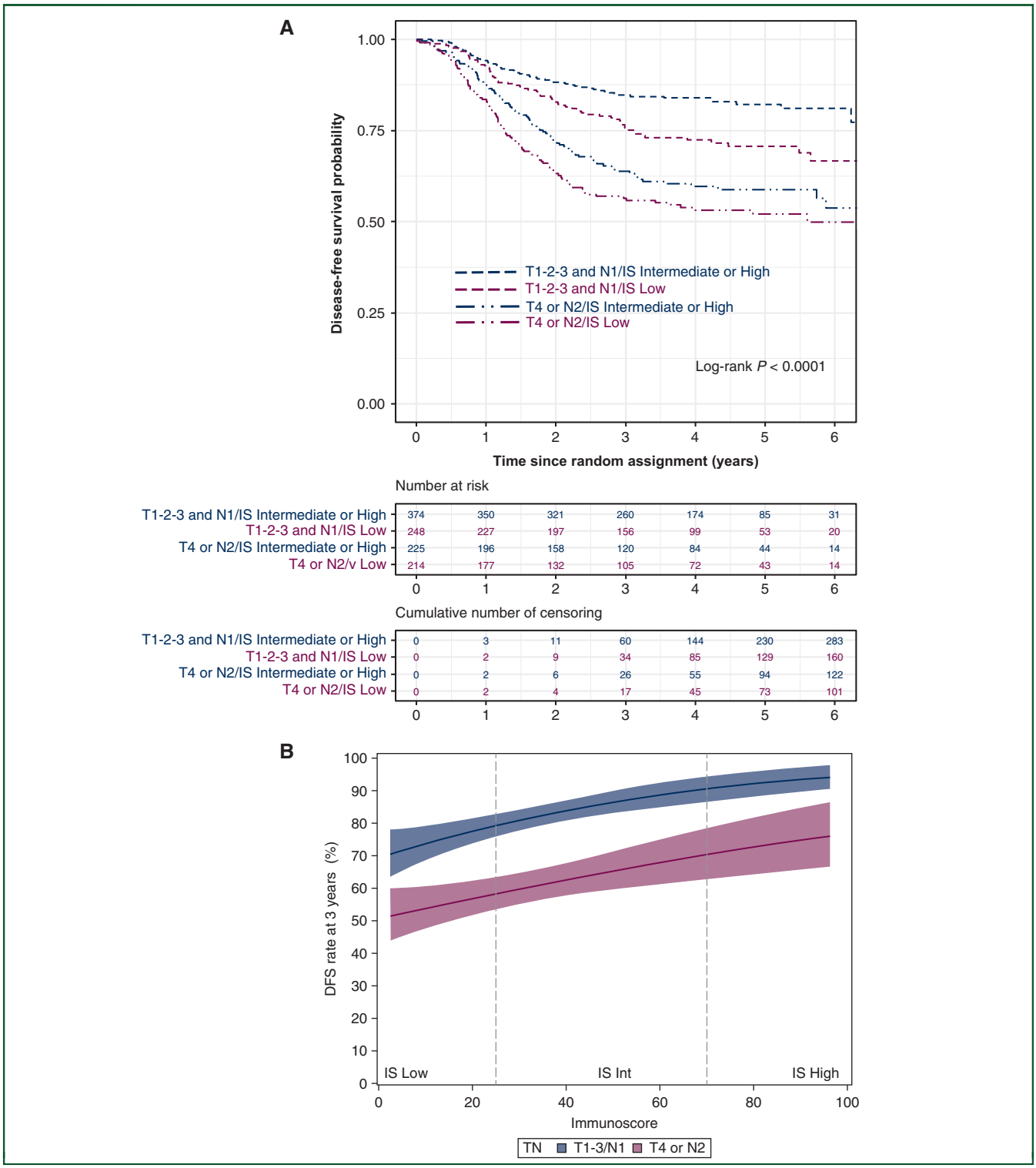


Figure 3. Impact of Immunoscore (IS) on disease-free survival (DFS) in low- and high-risk patients in the overall modified intention-to-treat population.
(A) Kaplan—Meier curve for DFS in clinically low-risk (T1—3/N1; dotted lines) and high-risk (T4 and/or N2; broken lines) patients, with IS Low (burgundy) and IS Int + High (blue). (B) The 3-year DFS in its continuous form according to IS in clinically low-risk (T1—3/N1; blue) and high-risk (T4 and/or N2; burgundy) patients.

patients treated with oxaliplatin-based chemotherapy (mFOLFOX6 or CAPOX), with a high level (Ib) of evidence according to the Journal National Cancer Institute (JNCI) guidelines.[13] In addition, patients with IS Int or IS High derived a significant benefit from the 6-month mFOLFOX6 regimen compared with the 3-month treatment, both in the clinically low (T1—T3, N1) and high-risk (T4 and/or N2) groups (secondary objective).

In this study, IS, reflecting the *in situ* density of the cytotoxic adaptive immunity (CD3+ and CD8+ T cells) of stage III CC, influenced the incidence of tumor recurrence and death. This finding confirms the clinical validity of IS

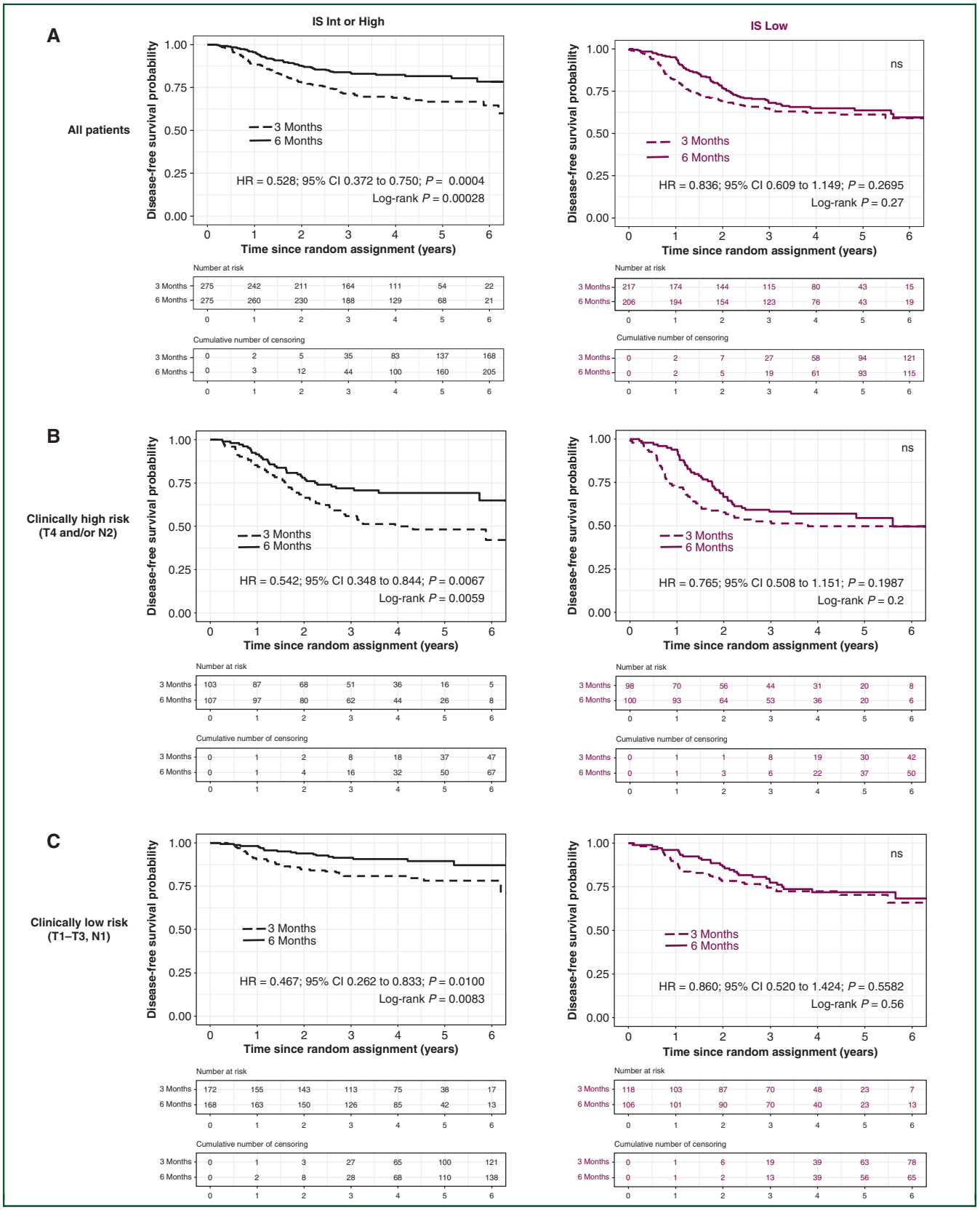


Figure 4. Impact of the 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) treatment duration on disease-free survival (DFS) according to Immunoscore (IS). Kaplan–Meier curves for DFS in IS Int + High and IS Low patients treated with mFOLFOX6 for 6 months (continuous line) or 3 months (dashed line) in (A) the whole population, (B) the clinically high-risk (T4 and/or N2) group, and (C) in the clinically low-risk (T1—T3 and N1) group. CI, confidence interval; HR, hazard ratio; NS, not significant.

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previously observed in our nonrandomized retrospective international validation study of 3547 stage I—III CC patients.[12] Moreover, this is in agreement with the prognostic value of IS observed in stage III CC patients ($n = 600$) of the FOLFOX arm in the prospective NCCTG N0147 clinical trial.[14,15] Besides, in the PETACC-8 trial, the *in situ* immune infiltrate (CD3+) assessment showed a concordant prognostic value of IS for recurrence and death in stage III CC patients treated with adjuvant FOLFOX.[10]

The feasibility, robustness, and reproducibility of IS are essential steps necessary for its integration in clinical practice. In this study, 85% of the samples with a preanalytic conformity were successfully determined by IS. The main causes of failure were torn tissue and a low signal intensity for old tumor blocks. In routine practice for prospective cases, the success rate exceeded 95% (data not shown). Importantly, we confirmed our previous observation[12] of an excellent concordance for the determination of a wide range of immune densities between centers.

One could expect that patients with IS Low, who were shown to have the highest risk of recurrence and/or death, would be the ones who benefit most from the 6-month mFOLFOX6 regimen compared with those with the 3-month therapy. Strikingly, this is not the case. On the contrary, a lack of benefit from the 6-month mFOLFOX6 therapy was shown in patients with IS Low. Patients with IS Low (43.6% of the study population) appeared to be doubly penalized by an increased risk of recurrence and/or death and the lack of benefit from longer duration of treatment. Only patients with IS Int or High (56.4% of the study population) gained from 6 months of mFOLFOX6 treatment as compared with 3 months of therapy. Importantly, clinically low-risk patients (T1—3, N1) with IS Int or High had the 3-year DFS of 80.8% when treated with the 3-month mFOLFOX6, which was significantly prolonged with the 6-month regimen (DFS of 91.4%). Thus, even low-risk (T1—3, N1) patients could effectively benefit from 6 months of mFOLFOX6, depending on the IS status.

Biological plausibility is important considering that the IDEA study did not show substantial differences between 3 and 6 months. The advantage of a longer duration of treatment only observed in IS patients (Int + High) would be counter-intuitive if there was no influence of quality of the immune component on the effectiveness of chemotherapy. Interestingly, a part of the oxaliplatin-based chemotherapy activity could be mediated by the immune components. Oxaliplatin is one of the few chemotherapeutic agents eliciting *bona fide* immunogenic cell death[16] that favors curative tumor-associated adaptive immunity.[17] We can hypothesize that immunogenic cell death-driven immunity can no longer operate in tumors classified as IS Low, reflecting *in situ* immunological defects such as a weak immunogenicity of the tumor[18] and/or immunosuppressive environment.[19] In addition, the 5-fluorouracil regimen favors tumor immune response by decreasing the number of myeloid-derived suppressor cells[20] and by increasing cytotoxic T-cell function.[21] This beneficial effect could be far less pronounced in IS Low patients, reflecting a weak natural cytotoxic T-cell function against the tumor.

A limitation of our study is that 90% of patients in the IDEA France study were treated with the mFOLFOX6 regimen, which precludes any robust conclusion for patients receiving CAPOX. In addition, the median follow-up of the overall mITT population was 4.3 years and therefore it is still impossible to analyze the IS impact on long-term treatment benefit. However, the Adjuvant Colon Cancer ENd PoinTs (ACCENT) group has confirmed the persistent good correlation between the 3-year DFS and 5-year overall survival with oxaliplatin-based chemotherapy.[22] Moreover, previous studies showed the significant positive impact of IS on both DFS and overall survival.[8,9,12]

The predictive value of IS needs now to be confirmed in FOLFOX- and/or CAPOX-treated patients in another cohort of the IDEA collaboration to validate the potential use of the IS test in guiding the choice of duration of adjuvant therapy.

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DISCLOSURE

FP reported receiving grants from Bristol-Myers and Squibb and HalioDx outside the submitted work; participation in Scientific Advisory Boards & Meetings for Bristol-Myers Squibb, Roche, Janssen, Merck, and Gilead. FP has patents associated with the immune prognostic biomarkers. TA reported participation in Scientific Advisory Boards & Meetings and receiving honoraria from Roche, Ventana, Sanofi, Bristol-Myers Squib, Clovis, MSD Oncology, Servier, Tesaro, Pierre Fabre, and HalioDx outside the submitted work. JT reported receiving honoraria from Merck, Roche, Amgen, Lilly, Sanofi, Sirtex, Servier, Baxalta, and Celgene; a consulting or advisory role for Roche, Merck KGaA, Amgen, Celgene, Lilly, Shire, Servier, and Sirtex; speakers' bureau for Servier, Amgen, Shire, Roche, Genentech, Sanofi, Merck, Lilly, and Sirtex. DV reported receiving honoraria from HalioDx, Janssen, and Celgene; a consulting or advisory role for Celgene, HalioDx, Janssen-Cilag, and Theradiag. CB reported

receiving honoraria and consulting fees from Roche, and sat on advisory boards of Bayer, Sanofi, and Servier. CL reported a consulting or advisory role for Celgene; travel, accommodation, or expenses from Roche and Merck. JB reported receiving honoraria from Roche, Bristol-Myers Squibb, Astra-Zeneca, Boehringer Ingelheim, and MSD; a consulting or advisory role for Roche, Bristol-Myers Squibb, AstraZeneca, Boehringer Ingelheim, and MSD; travel, accommodations, expenses from Roche and Bristol-Myers Squibb. JD reported expert testimony for Roche; travel, accommodations, expenses from Hospira. PL-P reported personal fees from Amgen and Merck; a consultant role for Amgen, Boehringer Ingelheim, AstraZeneca, and Pfizer. FH is a HalioDx full time employee, co-founder, and shareholder. AC is a HalioDx full-time employee and Shareholder. JG is the co-founder and chairman of the scientific advisory board of HalioDx; reported receiving grants from Perkin-Elmer, IO Biotech, MedImmune, AstraZeneca, Imcheck Therapeutics, and Janssen; participates in scientific advisory boards of Bristol-Myers Squibb, MedImmune, AstraZeneca, Novartis, Definiens, Merck Serono, IO Biotech, ImmunID, NanoString, Illumina, Northwest Biotherapeutics, Actelion, Amgen, Kite Pharma, Merck, and MSD; is a consultant for Bristol-Myers Squibb, Roche, GSK, Compugen, Mologen, and Sanofi; also has patents associated with the immune prognostic biomarkers. J-FE reported receiving honoraria from Roche, GSK, MSD, Amgen, and HalioDx; also has patents associated with the immune prognostic biomarkers. JH, FM, RBJ, RF, AK, LM, and MS have declared no conflicts of interest.

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