PD-L1 Expression and Tumor-Infiltrating Lymphocytes Define Different Subsets of MAPK Inhibitor-Treated Melanoma Patients


Abstract

Purpose: To evaluate the expression of tumor PD-L1 and changes in tumor-infiltrating lymphocyte (TIL) populations in patients with metastatic melanoma treated with targeted MAPK inhibitors.

Experimental Design: Ninety-three tumors were analyzed from 40 patients treated with a BRAF inhibitor alone (BRAFi; n = 28) or combination of BRAFi and MEK inhibitors (Combi; n = 12). Tumors were excised before treatment (PRE), early during treatment (EDT), and at progression (PROG). Immunohistochemical staining was performed for CD4, CD8, CD68, FOXP3, LAG3, PD-1, and PD-L1 and correlated with clinical outcome.

Results: Patients’ tumors that were PD-L1 positive at baseline showed a significant decrease in PD-L1 expression at PROG (P = 0.028), whereas patients’ tumors that were PD-L1 negative at baseline showed a significant increase in PD-L1 expression at PROG (P = 0.008) irrespective of treatment with BRAFi or Combi. Overall PD-L1 expression highly correlated with TIL immune markers. BRAFi-treated patients showed significant increases in CD4+, CD8+, and PD-1+ lymphocytes from PRE to EDT (P = 0.001, P = 0.001, P = 0.017, respectively), and Combi-treated patients showed similar increases in CD4+ and CD8+ lymphocytes from PRE to EDT (P = 0.017, P = 0.021).

Conclusions: The addition of MEKi to BRAFi did not result in significant reduction in immune infiltration in EDT biopsies. This provides support for conducting trials that combine MAPKi with immune checkpoint inhibitors in the hope of improving complete and durable response rates. PD-L1 expression at PROG on MAPK inhibitors varied according to baseline expression suggesting that combining MAPKi with immunotherapies concurrently may be more effective in patients with PD-L1 expression and TILs in baseline melanoma samples.

Introduction

Drugs inhibiting CTLA-4 on T cells (ipilimumab; ref. 1) or the MAPK pathway in BRAF-mutant melanoma ( vemurafenib, dabrafenib, cobimetinib, and trametinib) have significantly improved the overall survival (OS) of patients with metastatic melanoma (2–5). Further improvements in patient outcomes have been achieved by monoclonal antibodies (mAb) that target the programmed cell death receptor 1 (PD-1, CD279; refs. 6, 7).

PD-1 is expressed on a subset of activated T cells, as well as other immune cells (8, 9). Interaction of PD-1 on T cells with its ligands programmed cell death ligand 1 (PD-L1, CD274; refs. 10, 11) and programmed cell death ligand 2 (PD-L2, CD273; refs. 12, 13) negatively regulates antigen receptor signaling and inhibits immune responses. PD-L1 is constitutively expressed on subsets of T cells, B cells, macrophages, and dendritic cells (DC). It may be further upregulated by cytokines such as IFNγ released by CD4+ helper T cells (14), which is believed to result in feedback suppression and evasion of immune responses against the tumor (15, 16).

Results from the phase I clinical trials of the anti-PD-1 antibodies nivolumab (6) and pembrolizumab (MK3475; ref. 7) showed response rates of 28% and 38%, respectively, in patients with advanced-stage metastatic melanoma. Longer follow-up on patients in phase I studies treated with nivolumab has shown 1- and 2-year survival rates of 62% and 48%, respectively (17), and 1-year survival rate in patients treated with pembrolizumab of 69% (65% in ipilimumab-refractory patients and 74% in ipilimumab-naïve patients; ref. 18). Higher response rates and survivals were reported in the results of the phase I study of the combination of nivolumab and ipilimumab in patients with metastatic melanoma (19). Phase I studies with monoclonal antibodies against PD-L1 have also shown promising results with minimal toxicities (20). It is unknown whether there is potential for converting a subset of these patients, who fail either immunotherapy or targeted therapy alone into long-term responders by...
patients included in this study have been previously reported but in all the cases each biopsy was reanalyzed for the purposes of this study (22). Biopsied tumor specimens were collected from consenting patients before MAPKi inhibitor treatment (PRE), early during treatment with MAPKi inhibitor (EDT), and at disease progression (PROG), with Human Ethics Review Committee approval as part of the Treat Excise Analyze for Melanoma (TEAM) study at Melanoma Institute Australia. Clinical and follow-up details were collated and analyzed on all patients (Table 1). The size of the excised EDT tumor was measured with calipers before and early during treatment with MAPKi inhibitor therapy, and the percentage change in area (short × long axis) was calculated. CT scanning was carried out at 6 to 9 weekly intervals and response was evaluated using the RECIST guidelines (28). Progression-free survival (PFS) and overall survival (OS) were calculated from date of starting MAPKi to event (either progression or death) or until last follow-up (censored).

Assessment of TIL

Patient biopsies were fixed with 10% buffered formalin solution and processed into paraffin-embedded (FFPE) tissue blocks. Sections were cut from the FFPE blocks and hematoxylin and eosin (H&E) staining was performed. The density and distribution of TILs at both the tumor periphery and within the tumor were scored using a 4-tiered system, as previously described (22). The percentage of the tumor that had an infiltrate of lymphocytes was scored, and the average number of lymphocytes per HPF in a minimum of 4 representative HPF areas was given a semiquantitative score using a 4-tiered scale (0, no lymphocytes; 1, 1–10/HPF; 2, 11–50/HPF; 3, >50/HPF). A TILs grade was derived by multiplying the percentage of tumor which had an infiltrate by the scores for the average number of lymphocytes per HPF to attain a score from 0 to 300.

Immunohistochemistry

All immunohistochemical (IHC) staining was carried out on 4-μm-thick sections using an Autostainer Plus (Dako - Agilent Technologies) with appropriate positive and negative controls. Sections were baked for 60 minutes at 60°C in a dehydrating oven and heat-induced epitope retrieved in the PT link (Dako - Agilent technologies) using EnVision FLEX target retrieval solution for 20 minutes at 97°C and then cooled to room temperature in TBST wash buffer for 5 minutes. Slides were incubated with the following antibodies at the following dilutions: CD4 (Cell Marque-SP35) 1:100, CD8 (Cell Marque-SP16) 1:200, CD68 (Cell Marque-KP-1) 1:1,000, PD-1 (Cell Marque-MRQ-22/NAT105) 1:100, LAG3 (LSBio-17B4) 1:1,000, FOXP3 (Abcam-AB22510) 1:200, PD-L1 (Merck-22C3) 1:1,000. The Envision flex Mouse linker (K8022) was used to amplify the signal for PD-1 and PD-L1. Antibody detection used the Envision FLEX Kit (K8023) with a DAB chromagen for visualization according to the manufacturer's instructions (Dako- Agilent technologies). Slides were then counterstained with hematoxylin. For immune markers (CD4, CD8, CD68, PD-1, LAG3, and FOXP3), the percentage of tumor which had an infiltrate of lymphocytes or macrophages was estimated, and the average number of positive immune cells per HPF in a minimum of 4 representative HPF areas was determined using a semiquantitative 4-tiered scale used for scoring lymphocytes (0, no lymphocytes; 1, 1–10/HPF; 2, 11–50/HPF; and 3, >50/HPF) and macrophages (0, no macrophages; 1, 1–50/HPF; 2, 50–100/HPF; 3, >100/HPF).
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**Abbreviations:** BD, twice a day; Dab, dabrafenib; LN, lymph node; NR, not recorded; SQ, subcutaneous; TDS, three times a day; Tram, trametinib; Unk, unknown; Vem, vemurafenib.

*Previous single-agent dabrafenib. All patients were ECOG performance status = 0 at baseline apart from those with #, which were ECOG = 1.

Size at baseline and on biopsy was calculated using the product of the longest perpendicular diameters.
Lymphocyte and macrophage infiltrate scores were obtained by multiplying these scores to attain a score from 0 to 300.

For the PD-L1 antibody, the percentage of both tumor cells and immune cells (lymphocytes and macrophages) showing positive cytoplasmic membrane staining was determined and the intensity of staining was judged on a semiquantitative scale of 0–3+: no staining (0), weakly positive staining (1+), moderately positive staining (2+), and strongly positive staining (3+). An immunoreactive score (IRS) was derived by multiplying the percentage of positive cells with staining intensity to attain a score of 0 to 300, as previously described (22, 29–34). Positive PD-L1 tumor staining was defined as ≥1% tumor cell expression. The patterns of PD-L1 staining were also recorded. Four descriptive categories of PD-L1 positive staining were defined using a decision tree (Supplementary Fig. S4) based on the location of PD-L1–expressing tumor cells, the percentage of tumor cells staining positive (≥ or < 40%) and the ratio of TILs to PD-L1–expressing tumor cells [< (not proportional) or ≥ (proportional) 1:1] in the immediate vicinity of the PD-L1–expressing tumor cells. The descriptive patterns were: pattern 1: diffuse (≥40% tumor PD-L1) staining unassociated with a proportionate TILs infiltrate (Fig. 1A); pattern 2: focal (<40% tumor PD-L1) staining unassociated with a proportionate TILs infiltrate (Fig. 1B); pattern 3: diffuse (≥40% tumor PD-L1) staining associated with a proportionate TILs infiltrate (Fig. 1C); and pattern 4: focal (<40% tumor PD-L1) staining associated with a proportionate TILs infiltrate either at the periphery of the tumor (pattern 4A; Fig. 1D) or nonperipherally within the tumor (pattern 4B; Fig. 1E and F). In cases displaying more than one pattern, the predominant pattern was described. A subset of tumor samples deemed to be indeterminable for PD-L1 expression due to heavy melanin pigmentation was bleached for 24 hours with 10% H2O2 before PD-L1 staining. All slides were reviewed independently by at least 2 of 3 observers (H. Kakavand, R. Vilain, and R.A. Scolyer) who were blinded to clinical outcomes. Any discrepancies were resolved by consensus following review at a multiheader microscope.

Statistical analysis

Statistical analyses were conducted with "PASW Statistics 21" SPSS, IBM. Wilcoxon matched-pairs method was used to test for significant changes in immune markers between the different
biopsy time points (PRE, EDT, and PROG). Correlations between different immune markers were conducted using the Spearman rho test. Univariate survival analysis was carried out using the Kaplan–Meier method together with the log-rank (Mantel–Cox) test to calculate statistical significance. Cox regression analysis was used to generate univariate HRs, 95% confidence intervals (CI), and corresponding P values. Statistical significance was defined as a probability level < 0.05.

Results

Patients’ treatments and biopsies

Multiple tumor excision biopsies were available for analysis from 40 patients (Table 1). All patients had a PRE biopsy before initiating MAPKi therapy and 28 had paired EDT biopsies and 25 PROG biopsies. There were 28 patients (28 PRE, 18 EDT, 18 PROG) treated with a BRAFi (dabrafenib = 22 or vemurafenib = 6) and 12 patients (12 PRE, 10 EDT, 7 PROG) treated with Combi (dabrafenib and trametinib). There were no significant differences in clinical features [i.e., age, sex, V600E/K mutation status, M stage, Eastern Cooperative Oncology Group (ECOG), and lactate dehydrogenase (LDH)] at baseline between these 2 cohorts (Table 1). All patients were immunotherapy-naïve and 38 were MAPKi-naïve at baseline (patient 34 and 35 had received a BRAF inhibitor before Combi). Twenty-eight (70%) patients had a BRAF V600E mutation and 12 (30%) had a BRAF V600K mutation and corresponding P-values at baseline (patient 34 and 35 had received a BRAF inhibitor before Combi). Twenty-eight (70%) patients had a BRAF V600E mutation and 12 (30%) had a BRAF V600K mutation. There were 14 females and 26 males and the median patient age was 51 years (range, 23–80 years). Two patients achieved a complete response (CR), 25 had a partial response (PR), 12 had stable disease (SD), and one had progressive disease (PD) as their best RECIST response. The median follow-up period was 11 months (range, 2–50 months), with a median time to progression of 5.5 months (range, 1–15 months). At last follow-up, 29 patients had died of melanoma and 11 patients were still alive. Of those still alive 4 had not progressed on MAPKi therapy.

PD-L1 expression on melanoma and immune cells

PD-L1 expression showed marked intratumoral and intertumoral heterogeneity both in the percentage of positive staining melanoma cells and in the intensity of staining. Similar heterogeneity of PD-L1 staining was also observed on lymphocytes and macrophages (Supplementary Table S2). In the total cohort of 93 tumor samples taken at different time points, 55 (59%) displayed tumor immunoreactivity for PD-L1 (Supplementary Table S2 and Fig. S4). There was a significant positive correlation between tumor PD-L1 IRS and the density/distribution of TILs (r = 0.288, P = 0.005) as well as the density/distribution of CD4+ and CD8+, PD-1+, LAG3+ and FOXP3+ lymphocyte subsets in all 93 biopsies (r = 0.365, P = 0.0004; r = 0.351, P = 0.0006; r = 0.402, P = 0.0001; r = 0.593, P = 4 × 10−10; r = 0.353, P = 0.0006, respectively). There was also marked co-localization of tumor PD-L1 with intratumoral PD-1+ lymphocytes. In the 55 tumor PD-L1-positive biopsies, the median PD-L1 IRS was 5. Four (7.3%) cases showed diffuse (>40% tumor PD-L1) staining that was unassociated with a proportionate TILs infiltrate (pattern 1). 1 (1.8%) case showed focal (<40% tumor PD-L1) staining that was unassociated with a proportionate TILs infiltrate (pattern 2). 7 (12.7%) cases showed diffuse (>40% tumor PD-L1) staining associated with a proportionate TILs infiltrate (pattern 3), 16 (29.1%) cases showed peripheral focal (<40% tumor PD-L1) staining associated with a proportionate TILs infiltrate (Pattern 4A), and 27 (49.1%) cases showed nonperipheral (intratumoral) focal (<40% tumor PD-L1) staining associated with a proportionate TILs infiltrate (Pattern 4B, Fig. 1, Supplementary Table S1 and Fig. S4).

Effect of MAPK inhibitors on PD-L1 expression

There was no significant difference in the overall tumor PD-L1 IRS or positivity rates between the different treatment time points (PRE, EDT, and PROG). Positive tumor PD-L1 expression was identified in 24 of 40 (60%) PRE biopsies, 15 of 28 (54%) EDT biopsies, and 16 of 25 (64%) PROG biopsies. There were no significant differences in staining patterns between the PRE, EDT, and PROG time points; however, the predominant pattern of staining in the biopsies was nonperipheral (intratumoral) focal PD-L1 positivity associated with a proportionate TILs infiltrate (pattern 4B, Supplementary Table S1).

There was no significant change in lymphocyte or macrophage PD-L1 expression between the various time points and treatment regimens (Supplementary Table S2). Overall, there was no significant change in tumor PD-L1 expression from PRE to EDT in patients’ tumors whether they were PD-L1 positive (n = 20) or negative (n = 8) at baseline. However, additional patient subgroups further defined by change in PD-L1, TILs, PD-1+ lymphocytes from PRE to EDT were also examined in an attempt to identify associations with clinical outcome measures (see below; Supplementary Fig. S1). There was no significant change in PD-L1 expression from PRE to PROG. However, in patients with PD-L1-positive tumors at baseline that had a matched PROG sample (n = 13), PD-L1 expression significantly decreased from PRE to PROG (P = 0.028; Fig. 2A). This coincided with a significant decrease in TILs (P = 0.035) from PRE to PROG. In contrast, PD-L1 expression increased significantly from PRE to PROG (P = 0.008; Fig. 2B) in those with PD-L1-negative tumors at baseline that had a matched PROG sample (n = 12). This coincided with a significant increase in TILs (P = 0.008) and PD-1+ lymphocytes (P = 0.044) from PRE to PROG.

BRAFi monotherapy and combination MAPKi therapy had similar effects on TIL influx in sequential biopsies

In the total cohort of patients (n = 40), there was a significant increase in TILs from PRE to EDT (P = 0.000063), which then decreased significantly from EDT to PROG (P = 0.045). There was no significant difference in TILs between the PRE and PROG biopsies (P = 0.594). There was a significant increase in the CD4+, CD8+, and PD-1+ intratumoral lymphocyte subsets (P = 0.00004, P = 0.00003, P = 0.007, respectively) from PRE to EDT. However, the decreases from EDT to PROG in these markers were not significant. There was no significant change in FOXP3+ regulatory T lymphocytes and LAG3+ lymphocytes at any time point (Supplementary Fig. S2), with levels of expression being variable at PRE, EDT, and PROG in different patients. There was also no significant change in CD68-expressing macrophages at any time point in either the BRAFi-treated or Combi-treated patients. In patients treated with Combi, there were significant increases in CD4+ and CD8+ intratumoral lymphocytes from PRE to EDT (P = 0.011 and P = 0.013, respectively; Fig. 3D and E), which were similar to the increases seen in patients treated with BRAFi (P = 0.001 and P = 0.001, respectively; Fig. 3A and B). However, there was a significant increase in PD-1+ intratumoral lymphocytes from PRE to EDT in patients treated with BRAFi (P = 0.001 and P = 0.001, respectively; Fig. 3A and B).
The expression of the immune markers CD4, CD8, PD-1, LAG3, and FOXP3 in lymphocytes and tumor PD-L1 IRS scores at any time point as well as the changes from PRE to EDT, PRE to PROG, and EDT to PROG did not show any significant correlation with any clinical parameters such as site of PRE biopsy, best RECIST response, EDT tumor response, number of days to the EDT biopsy, change in the EDT tumor size, PFS, and OS. In contrast, increased TILs at PRE biopsy was associated with a better RECIST response ($P = 0.002$) but not improved PFS or OS.

Analysis based on patients’ PD-L1 status at baseline and the change in PD-L1, TILs, and PD-L1+ lymphocytes from PRE to EDT revealed subgroups with marked heterogeneity in response to MAPKi therapy and survival outcome measures (Supplementary Fig S1). Furthermore, there was no difference in clinical outcome between those with an increase in PD-L1 expression from PRE to PROG and those with a decrease. Cox regression analysis showed no significant predictive value of baseline immune markers (CD4, CD8, PD-1, LAG3, and FOXP3) and baseline tumor PD-L1 expression for PFS or OS (Supplementary Fig S3). A larger RECIST response was associated with longer PFS and OS (HR, 0.47; 95% CI, 0.23–0.98; $P = 0.04$ and HR, 0.39; 95% CI, 0.17–0.86; $P = 0.02$, respectively).

**Discussion**

PD-L1 expression on melanoma cells is believed to be induced mainly by T-cell responses mounted against the tumor and to be an important adaptive response that allows escape from immune attack (35, 36). In some melanomas, PD-L1 expression may also be driven through oncogenic pathways as has been described in non–small cell lung carcinoma with EGFR mutations (37) or be the result of activation of pre-existing or acquired treatment resistance pathways in melanoma that are upregulated during treatment with MAPK inhibitors (23, 38). The present study assessed unique sequential biopsies taken before treatment, early during treatment, and at time of progression from patients treated with BRAFi or Combi and was focused particularly on changes in PD-L1 expression as a result of MAPK inhibition and whether the addition of an MEK inhibitor to a BRAF inhibitor decreased the TIL infiltrate seen in patients treated with a BRAF inhibitor alone.

This study extends previous preliminary results that intratumoral infiltrates in patients with metastatic melanoma treated with MAPKi by using IHC to assess PD-L1 expression in melanoma and changes in intratumoral PD-L1+ lymphocytes in greater detail. The results show that PD-L1 expression on melanoma cells was increased in a subset of patients progressing on treatment with BRAFi or Combi who had no PD-L1 expression and low TILs at baseline (“non-immunogenic” group). The increase in PD-L1 expression in this subset correlated with an influx of TILs in the progressed samples. These findings are consistent with induction of an immune response against the melanoma cells during treatment with MAPKi inhibitor, which could lead to an increased expression of PD-L1 at progression as a mechanism of immune evasion. In contrast, patients who had high PD-L1 expression and high TILs at baseline (“immunogenic” group) showed significant decreases in both PD-L1 and TILs in the samples taken at PROG. In these patients, the decrease in PD-L1 expression in the PROG samples may be due to the absence of TILs and thus no stimulus for induction of PD-L1 on tumor cells or it may reflect induction of resistance pathways in melanoma and release of cytokines that inhibit T-cell responses as well as promoting tumor proliferation. There was no significant increase in PD-L1 expression from PRE to EDT; even though there was an overall increase in TILs at EDT in the study cohort, these were not specifically PD-L1-expressing lymphocytes, which produce cytokines that induce PD-L1 expression. There was no significant change in lymphocyte or macrophage PD-L1 expression between the various time points and treatment regimens, suggesting that MAPKi inhibitors do not alter the presence of PD-L1–expressing immune cells in these biopsies. Furthermore, there was marked heterogeneity of response and outcome measures between the patients irrespective of the expression of any of the immune markers analyzed early during treatment. Nevertheless, it
appeared that the patient subgroup with the best RECIST responses were PD-L1 positive at baseline and showed a decrease in PD-L1 expression and an increase in TILs and PD-1⁺ lymphocytes from baseline to early during treatment. This may indicate a decreased ability of the melanoma cells to inhibit the effect of the differentiated PD-1⁺ lymphocytes through binding with PD-L1, thereby prolonging the latter’s cytotoxic activity.

Our findings extend those of Frederick and colleagues who assessed, in a smaller cohort of patients (n = 11), the mRNA levels of PD-L1 in fresh-frozen tumors taken at baseline, early during treatment, and at progression in patients receiving MAPK inhibitors and validated their findings using IHC in matched patients’ samples (24). They showed a significantly increased level of the transcript in patients’ early during treatment biopsies compared with baseline biopsies. In our study, we used the highly specific PD-L1 antibody (22C3) to clearly detect the clinically relevant (39) membranous staining on the tumor cells. Whereas Frederick and colleagues showed a significant increase in PD-L1 expression and TILs from PRE to EDT in the BRAFi-treated group (A–C), there was no significant change in PD-1⁺ lymphocytes in the Combi-treated group (F).

The inclusion of patients in this study that had been treated with both dabrafenib and trametinib provided us an opportunity to determine whether differences in PD-L1 expression and TILs might contribute to the improved PFS and OS seen in patients treated with the combination compared with those treated with dabrafenib alone. This was particularly important as previous studies have shown that MEK inhibitors are potentially immunosuppressive (40, 41). We found no evidence for marked immunosuppressive effects in the TILs in the sequential biopsies taken from patients treated with Combi, which appeared comparable to those in patients treated with BRAFi monotherapy. A significant increase in PD-1⁺ lymphocytes at EDT was observed in patients treated with BRAFi alone but not in the Combi-treated group. PD-1 expression on lymphocytes is a mechanism of early terminal differentiation and our results could indicate a potential defect in the differentiation of T cells in the Combi treated patients. Alternatively, it could be that there was a lack of power in our study to identify significant differences in PD-1⁺ expressing lymphocytes as there were fewer Combi-treated patients (n = 12) compared with patients treated with BRAFi alone (n = 28). LAG3, another checkpoint inhibitor, is believed to reflect decreased effector T-cell function as a result of activation of the egr2 transcription factor (42). Interestingly, we observed no substantial differences in LAG3-expressing T cells in patients treated with
BRAFi or Combi. Activation of the MAPK pathway is known to be strongly immunosuppressive (43), and its reactivation is a common mechanism for failure of treatment with BRAFi (38, 44). Induction of suppressor T cells has also been implicated as a cause of tumor progression but we did not detect significant changes in FOXP3+ lymphocytes at any time point with either BRAFi or Combi.

In conclusion, our data showing the addition of MEKi to BRAFi did not result in significant reduction in immune infiltration of CD4+ and CD8+ lymphocytes into the early during treatment biopsies, which taken together with the results of a recent study demonstrating the predictive value of baseline peritumoral CD8+ lymphocytes for response to PD-1 inhibitors (25), provides support for conducting trials that combine MAPKi with immune checkpoint inhibitors in the hope of improving complete and durable response rates. They also provide a rationale for the concurrent administration of targeted MAPKi inhibitors with PD-1/PD-L1 blockade in patients with BRAF-mutant metastatic melanoma. Furthermore, assessment of PD-L1 expression and TILs infiltrates may identify a subgroup of patients who respond better to the combination of targeted MAPKi inhibitor and immune checkpoint inhibitors.

Discord of Potential Conflicts of Interest

A.M. Menzies reports receiving speakers bureau honoraria from GlaxoSmithKline and Roche. R.F. Kefford reports receiving speakers bureau honoraria from Bristol-Myers Squibb, GlaxoSmithKline, and Merck and is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, Merck, Novartis, and Roche. G.V. Long reports receiving speakers bureau honoraria from Merck and is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, Merck, Novartis, and Roche. No potential conflicts of interest were disclosed by the other authors.

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