Clinical Cancer Research

Circulating Cytokines Predict Immune-Related Toxicity in Melanoma Patients Receiving Anti-PD-1–Based Immunotherapy

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Abstract

Purpose: Combination PD-1 and CTLA-4 inhibitor therapy has dramatically improved the survival of patients with advanced melanoma but is also associated with significant immune-related toxicities. This study sought to identify circulating cytokine biomarkers of treatment response and immune-related toxicity.

Experimental Design: The expression of 65 cytokines was profiled longitudinally in 98 patients with melanoma treated with PD-1 inhibitors, alone or in combination with anti-CTLA-4, and in an independent validation cohort of 49 patients treated with combination anti-PD-1 and anti-CTLA-4. Cytokine expression was correlated with RECIST response and immune-related toxicity, defined as toxicity that warranted permanent discontinuation of treatment and administration of high-dose steroids.

Results: Eleven cytokines were significantly upregulated in patients with severe immune-related toxicities at baseline (PRE) and early during treatment (EDT). The expression of these 11 cytokines was integrated into a single toxicity score, the CYTOX (cytokine toxicity) score, and the predictive utility of this score was confirmed in the discovery and validation cohorts. The AUC for the CYTOX score in the validation cohort was 0.68 at PRE [95% confidence interval (CI), 0.51–0.84; $P = 0.037$] and 0.70 at EDT (95% CI, 0.55–0.85; $P = 0.017$) using ROC analysis.

Conclusions: The CYTOX score is predictive of severe immune-related toxicity in patients with melanoma treated with combination anti-CTLA-4 and anti-PD-1 immunotherapy. This score, which includes proinflammatory cytokines such as IL1a, IL2, and IFNα2, may help in the early management of severe, potentially life-threatening immune-related toxicity.

See related commentary by Johnson and Balko, p. 1452

Introduction

The last 5 years have seen remarkable improvements in the survival of patients with advanced melanoma due to the use of immune checkpoint therapies that promote T-cell–mediated antitumor activity (1, 2). Immune checkpoint inhibitors targeting the cytotoxic T-lymphocyte antigen-4 (CTLA-4; ipilimumab) and programmed death-1 (PD-1; nivolumab and pembrolizumab) receptors produce durable responses and prolong the survival of patients with melanoma (3–5), and have now entered adjuvant therapy.

Circulating biomarkers of response to anti-PD-1–based therapy have been widely investigated. For instance, circulating tumor DNA, frequency of activated monocytes ($CD14^+$ $CD16^-$ $HLA\text{-}DR^{hi}$), and neutrophil counts have each been shown to predict progression-free and overall survival in patients treated with immune checkpoint inhibitors (6–8). In contrast, there have been few studies exploring biomarkers that predict risk of immune-related adverse events (irAE; reviewed in ref. 9), despite grade 3/4 irAEs occurring in over 50% of patients treated with combination immune checkpoint inhibitors (10, 11). Early recognition and intervention is critical for severe irAEs, and many patients require treatment interruption, discontinuation, and/or immunosuppressive agents such as high-dose steroids, infliximab, or mycophenolate (12).

Cytokines are important regulators of host immune activity; they promote the recruitment of immune cells into the tumor microenvironment (13–17) and induce the expression of immune checkpoint receptors, including TIM-3 and PD-1 (18). Consequently, these soluble mediators may serve as predictive biomarkers of immunotherapy response and/or immune-mediated toxicity. For instance, baseline serum levels of TGFβ and IL10 correlated with clinical outcome, and elevated baseline IL17 levels predicted grade 3 colitis in patients with melanoma treated with neoadjuvant ipilimumab ($n = 35$; ref. 19). These findings are yet

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Translational Relevance

The identification of blood-based biomarkers that can predict immunotherapy toxicity and response remains a crucial but unmet need. This study explored the utility of circulating plasma cytokines in predicting immune-related toxicity and treatment response in patients with melanoma treated with anti-PD-1–based immunotherapy, alone or in combination with anti-CTLA-4. Plasma cytokine levels were generally unchanged with immunotherapy, indicating that analysis of either baseline (PRE) or early during treatment (EDT) plasma may prove useful as a stable biomarker of toxicity. We defined a toxicity score (CYTOX) that includes the expression of 11 circulating cytokines, which was significantly higher in patients with severe immune-related toxicity, at PRE and EDT. We validated the predictive utility of the CYTOX score in an independent cohort of patients treated with combination PD-1 and CTLA-4 inhibitors. These findings have important clinical implications in the prediction and early management of severe, potentially life-threatening immune-related toxicity.

to be validated, however, and the role of circulating cytokines in predicting immune-related toxicity remains unexplored.

In this study, we analyzed the expression of 65 cytokines in longitudinal plasma samples collected prior to therapy and during treatment from patients with melanoma treated with PD-1 inhibitors (nivolumab or pembrolizumab), alone or in combination with the CTLA-4 inhibitor ipilimumab. Cytokine expression was correlated to the development of immune-related toxicity. Although cytokine levels displayed large interpatient variation, there was limited intrapatient variability over the course of treatment. Importantly, elevated levels of 11 circulating cytokines at baseline (PRE) and early during treatment (EDT) were significantly associated with the development of high-grade immune-related toxicity. The integration of these 11 cytokines into a single toxicity score (CYTOX) also predicted immune-related toxicity in an independent cohort of patients with melanoma treated with combination PD-1 and CTLA-4 inhibitors.

Materials and Methods

Patients and treatment

Patients with stage III or IV melanoma treated with immunotherapy at the Melanoma Institute Australia (Wollstonecraft New South Wales, Australia) and Westmead Hospital (Westmead New South Wales, Australia) between November 2012 and February 2018 were included in this study. The study was conducted in accordance with the Declaration of Helsinki and the National Health and Medical Research Council of Australia's National Statement on Ethical Conduct in Human Research. Written informed consent was obtained from all patients under approved Human Research Ethics Committee protocols from the Royal Prince Alfred Hospital (Protocol X15-0454 and HREC/11/RPAH/444). Patients with melanoma were treated with either anti-PD-1 monotherapy (pembrolizumab or nivolumab), or anti-PD-1 in combination with anti-CTLA-4 (ipilimumab). Dosing regimens were according to FDA-approved doses or administered according to the schedule in the ABC, MK3475-001, MK3475-006, MK3475-029, CA209-742, CA209-401, CheckMate-511, and OpACIN-neo clinical trials (NCT02374242, NCT01295827, NCT02083484, NCT02089685, NCT02905266, NCT02599402, NCT02714218, and NCT02977052; refs. 20, 21).

Disease characteristics and clinical assessment

Patient demographics and clinicopathologic features including sex, age, baseline lactate dehydrogenase (LDH) levels, and American Joint Committee on Cancer (8th edition) M stage were collected. Baseline disease tumor burden was determined by the sum of the product of bidimensional diameters (SPOD) for every metastasis $\geq$5 mm in long axis ($\geq$15 mm short axis for lymph nodes). A SPOD of $\leq$1,000 mm^2 was considered low-disease burden and >1,000 mm^2 was considered high-disease burden (6). Investigator-determined objective response was assessed radiologically with CT and MRI of the brain at 2–3 monthly intervals using the RECIST 1.1 criteria (22). Patients with a complete response (CR) or partial response as best response were classified as responders, and those with stable disease or progressive disease were classified as nonresponders. Follow-up duration was calculated from the date of first dose of systemic therapy to the following three dates: date of death, loss to follow-up, or May 31, 2018.

Toxicity data were categorized according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. Severe irAEs were defined as requiring dose interruption and the use of high-dose steroids (prednisolone dose $\geq$50 mg/day equivalent), or other immunomodulatory drugs such as infliximab and mycophenolate. We included only severe irAEs that developed within 6 months of starting treatment, as delayed toxicity may be difficult to predict using pretreatment and early during treatment cytokine levels. Treatment of irAEs was in line with standard guidelines and at the treating clinician's discretion. Endocrinopathies requiring hormonal replacement including uncomplicated hypophysitis were not considered severe toxicities. Patients who did not have severe toxicity according to the above description or no toxicity were categorized as "no severe irAEs."

Plasma samples

Peripheral blood samples from patients were collected prospectively at baseline (PRE, collected 0–29 days prior to therapy initiation), and at regular intervals during therapy, including early (EDT; week 1–6 after therapy initiation), MID (week 7–11 after therapy), and LATE (week 12–18 after therapy). Blood samples (~10 mL) were collected in EDTA vacutainer tubes (BD Vacutainer blood collection tubes) and processed within 4 hours of collection. Samples were centrifuged at 1,500 rpm (800 $\times$ g) for 15 minutes at room temperature for plasma collection, followed by a second centrifugation at 4,100 rpm (1,600 $\times$ g) for 10 minutes for further plasma clearance. Clarified plasma samples were stored at -80°C.

Multiplex cytokine/chemokine assay

Undiluted plasma samples were profiled using the 65-plex Human Cytokine/Chemokine Discovery Assay (catalog no: HD65; Eve Technologies). The 65-plex discovery assay utilized 150 μL of undiluted plasma per run and each run was performed in duplicate; duplicates did not vary by more than 4% (data not shown). Fluorescence intensity values were derived from the discovery assay and are in direct proportion to reflect the amount of proteins in the samples (23). Fluorescence values were

normalized to account for potential batch and plate effects as described previously (24).

Statistical analysis

Fluorescence intensity values were $\log_2$ transformed to achieve homogeneity of variance and to produce a less skewed distribution for analysis. Differential cytokine expression was carried out using the Morpheus online analysis tool (https://software.broadinstitute.org/morpheus; Broad Institute, Cambridge, MA). Differentially expressed cytokines were selected using the t test with an FDR-adjusted $P \leq 0.05$ and $\log_2$ fold change ($\log_2$ FC) > 0.7 as cutoff criterion. The sum of 11 cytokines differentially expressed in patients with severe irAEs compared with patients with no severe irAEs was defined as the CYTOX score. ROC analyses were used to assess the performance of the CYTOX score in a separate validation cohort. ROC analyses and curves were generated in GraphPad Prism (Version 7.02). Kaplan–Meier survival analysis was performed for each cytokine using the log-rank test, and RECIST response assessed using Fisher exact test. Patients were classed into two groups, either above or below the median cytokine value and the corresponding association with survival and response tested.

Results

Patient and sample characteristics

The discovery cohort comprised 98 patients with unresectable stage III or IV melanoma; 40 patients were treated with anti-PD-1 monotherapy (cohort 1, 40/98) and 58 were treated with combination PD-1 and CTLA-4 inhibitor therapy (cohort 2, 58/98). Median follow-up was 25.2 months (range 2.4–45.1 months) in cohort 1 and 36.3 months (range 3.1–40.5 months) in cohort 2, with 14 (35%) patients and 47 (81%) patients alive, respectively, at time of analysis. The two treatment groups were similar for sex, age, LDH, and baseline disease volume with more patients with stage M1c in cohort 1 compared with cohort 2 (85% vs. 59%; $P < 0.01$; Fisher exact test; Table 1). The objective response rate was 16 of 40 (40%) in cohort 1 and 47 of 58 (81%) in cohort 2, and with 5 of 58 (9%) patients undergoing CR in cohort 2. An independent validation cohort (cohort 3) consisting of 49 patients receiving combination anti-PD-1 and anti-CTLA-4 therapy was also included, and clinical characteristics are shown in Table 1. The median follow-up was 14.5 months in this cohort, and 43 (88%) patients were alive at time of analysis.

A total of 309 plasma samples were collected from cohorts 1 and 2, including a PRE sample for all 98 patients, and longitudinal samples throughout the course of treatment (EDT, MID, and LATE; Supplementary Table S1). A total of 98 plasma samples were collected from cohort 3, including 49 PRE and 49 EDT samples.

Cytokine profiles display significant interpatient but limited intrapatient alterations

We initially examined the baseline expression of 65 circulating cytokines in discovery cohorts 1 and 2 to explore the extent of interpatient variation. PRE plasma analysis revealed that the majority of cytokines (46/65; 71%) were expressed at relatively low levels, with median relative fluorescence units (RFU) of less than 100. There was large interpatient variation for most cytokines, with a coefficient of variation (CV) of more than 200% in 34 of 65 (52%) cytokines. IL5, IL17A, IL20, and MIP-1α demonstrated the most variation, with a CV of more than 300% (Supplementary Fig. S1).

The temporal profile of cytokine expression was interrogated by examining longitudinal plasma samples during treatment. Unbiased clustering based on cytokine expression profiles showed that the majority of plasma samples remained clustered by patient, indicating limited alteration in patient cytokine levels during the course of treatment (Supplementary Fig. S2). Accordingly, comparison of patient-matched PRE–EDT plasma pairs ($n = 95$) or PRE–EDT–MID ($n = 14$) in cohorts 1 and 2, showed no significant change over time in the expression of the 65 cytokines (data not shown).

Elevated expression of circulating cytokines in patients with severe immune-related toxicity

The association of circulating cytokines with the development of severe irAEs was initially examined in patients treated with combination immunotherapy (cohort 2) as this treatment produces substantially higher toxicity than anti-PD-1 monotherapy (25). Importantly, therapy-associated toxicity in patients treated with combination anti-PD-1 and anti-CTLA-4 did not correlate with treatment response (Supplementary Table S2). The

Table 1. Patient and disease characteristics

	Discovery		Validation
Characteristics	**Cohort 1 anti-PD-1 ($n = 40$)**	**Cohort 2 anti-CTLA-4 + anti-PD-1 ($n = 58$)**	**Cohort 3 anti-CTLA-4+ anti-PD-1 ($n = 49$)**
Age (years)			
Median (range)	67.5 (34–90)	57.5 (31–80)	59 (27–78)
Sex, *n* (%)			
Male	26 (65)	40 (69)	29 (59)
Female	14 (35)	18 (31)	20 (41)
LDH (%)			
≤1× ULN	26 (65)	38 (66)	37 (73)
>1× ULN	14 (35)	20 (34)	12 (27)
Disease volume (%)			
SPOD ≤1,000 mm^2	14 (35)	27 (47)	21 (43)
SPOD >1,000 mm^2	26 (65)	31 (53)	28 (57)
Stage (%)			
III	—	—	5 (10)
M1a or M1b	6 (15)	24 (41)	16 (33)
M1c	34 (85)	34 (59)	28 (57)

Abbreviation: ULN, upper limit of normal.

Table 2. Toxicity profiles of cohort 2 patients on combination immunotherapy

Timing of toxicity onset	Toxicity	No. of patients	Median time to onset Weeks (range)
<6 months ($n = 21$)	Colitis	6	8 (4–15)
	Severe dermatitis	5	1.7 (0.7–8.1)
	Autoimmune hepatitis	5	5 (2–12)
	Pneumonitis	2	13 (5–22)
	Pancreatitis	2	10 (9–11)
	Rheumatologic	1	1
≥6 months ($n = 3$)	Colitis	1	27
	Pancreatitis	1	48
	Optic neuritis	1	104
—	No immune-associated toxicity	27	—

clinicopathologic variables sex, age, LDH, stage, and baseline disease volume (Table 1) were also not associated with toxicity (data not shown).

Of the 58 patients in cohort 2, 52 patients had an EDT blood sample available within 3 weeks of therapy initiation. One patient was already on high-dose steroids for autoimmune hepatitis at the time EDT blood sample was taken and was excluded from this analysis. The remaining 51 patients were assessed to determine whether cytokine expression could predict severe immune-related toxicity, defined as irAEs that developed within 6 months and required dose interruption and the use of high-dose steroids (prednisolone dose ≥50 mg/day equivalent), or other immunomodulatory agents. The median time between EDT blood draw and onset of toxicity was 4 weeks (range 0.7–21 weeks).

Of the 51 patients, 24 (48%) had severe irAEs that required intervention. The onset of irAEs within 6 months of starting treatment occurred in 21 of 24 patients; 5 had autoimmune hepatitis, 6 had colitis, 5 had severe dermatitis, 2 had pneumonitis, 2 had pancreatitis, and 1 had an exacerbation of preexisting polymyalgia rheumatica. Late-onset immune-related toxicity occurred in 3 patients at 6.2, 11, and 24 months, with colitis, pancreatitis, and optic neuritis, respectively (Table 2).

We examined whether cytokines were differentially expressed in patients with severe irAEs compared with patients showing no severe irAEs using the Morpheus *t* test tool (Broad Institute, Cambridge, MA). We identified 17 cytokines at PRE and 16 cytokines at EDT that were differentially upregulated in patients with severe toxicities (FDR-adjusted $P \leq 0.05$ and $\log_2$ FC > 0.7; Supplementary Tables S3 and S4). Eleven of these differentially upregulated cytokines (G-CSF, GM-CSF, Fractalkine, FGF-2, IFNα2, IL12p70, IL1a, IL1B, IL1RA, IL2, and IL13) were elevated at both PRE and EDT time points (Fig. 1).

Cytokine panel is predictive of irAEs in melanoma

The 11 cytokines associated with severe toxicity were integrated into a single toxicity score, the CYTOX score, and the predictive utility of this score was evaluated in the discovery cohort 2 and an independent validation cohort 3 using ROC analysis. The AUC for the CYTOX score to discriminate severe toxicity was 0.78 [95% confidence interval (CI), 0.65–0.91; $P = 0.0009$] at PRE and 0.77 (95% CI, 0.63–0.90; $P = 0.0014$) at EDT for cohort 2 (Fig. 2; Table 3). Similar predictive performance was observed in the validation cohort 3. The AUC for the CYTOX score was 0.68 at PRE (95% CI, 0.51–0.84; $P = 0.037$) and 0.70 at EDT (95% CI, 0.55–0.85; $P = 0.017$; Fig. 2; Table 3).

Association of cytokine expression with response to immune checkpoint inhibition

We also investigated whether baseline cytokine expression was associated with patient response and survival to immunotherapy. When dichotomized around the median, TRAIL, MCP-1, and IL2 in patients treated with anti-PD-1 monotherapy (cohort 1), and TNFα, IL8, and IP-10 in patients receiving combination anti-CTLA-4 and anti-PD-1 (cohort 2) were each significantly associated with overall survival (Supplementary Table S5). Expression of IL2, IP-10, MCP-4, TARC, and ENA78 were also significantly associated with RECIST response in cohort 1 (Supplementary Table S6), but no significant association with response was found in cohort 2. Similarly, no cytokines were associated with response in validation cohort 3, and only SDF-1 was associated with survival in response to combination immunotherapy in the validation cohort (Supplementary Table S5).

Discussion

The combination of PD-1 and CTLA-4 inhibitors shows greatest efficacy but is associated with significant autoimmune toxicities, with over 50% of patients developing grade 3/4 irAEs (10, 11) that necessitate treatment interruption, discontinuation, and/or immunosuppression (12). Despite the seriousness of irAEs, there are limited reports on biomarkers that predict the risk of immune-related toxicity in patients treated with immunotherapy.

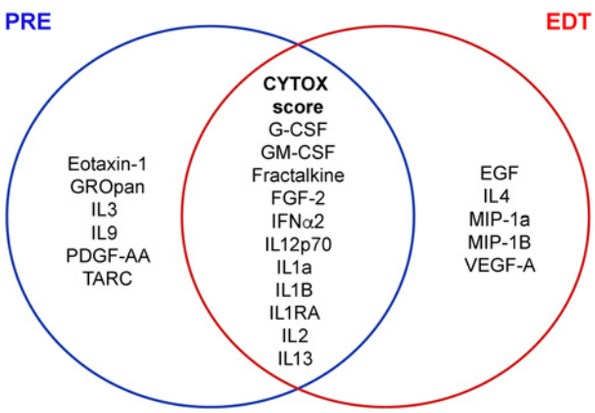

Figure 1.
Venn diagram of differentially expressed cytokines at PRE and EDT. Cytokines that were significantly elevated in patients with severe irAEs (FDR ≤ 0.05; $\log_2$ FC > 0.7) are shown. Cytokines elevated collectively at PRE and EDT were selected as the CYTOX score.

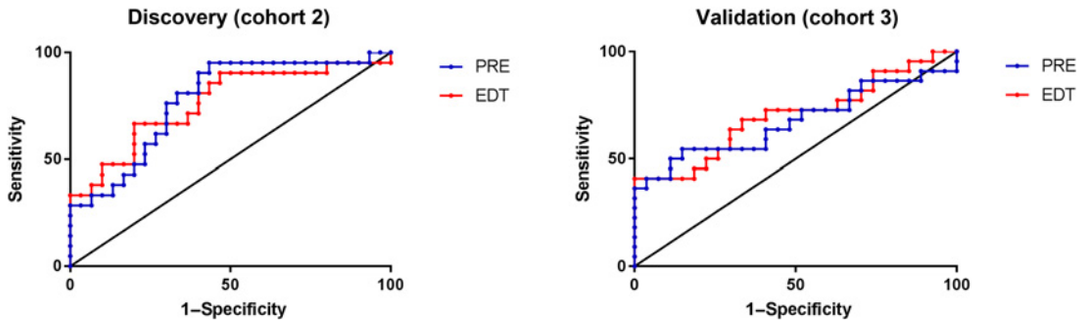

Figure 2.

ROC curve analysis of the 11-cytokine panel (CYTOX score) in the discovery cohort 2 and validation cohort 3. The sum of the 11-cytokine panel (G-CSF, GM-CSF, Fractalkine, FGF-2, IFNα2, IL12p70, IL1a, IL1B, IL1RA, IL2, and IL13) was compared using a ROC curve analysis between patients with severe irAEs and those with no severe irAEs at PRE and EDT in the discovery and validation cohorts.

Circulating cytokines such as decreased IL10 and increased IL17 levels have been associated with irAEs in patients with melanoma following treatment with ipilimumab (19, 26). Despite these findings, there are currently no validated biomarkers that can identify patients who are at risk of developing irAEs, or lead to the detection of toxicities prior to clinical manifestation. In this study, we demonstrated that elevated circulating cytokine levels are predictive of severe immune-related toxicity in patients with melanoma treated with combination anti-CTLA-4 and anti-PD-1 immunotherapy.

Elevated expression of 11 cytokines (G-CSF, GM-CSF, Fractalkine, FGF-2, IFNα2, IL12p70, IL1a, IL1B, IL1RA, IL2, and IL13) was strongly associated with severe irAEs that required intervention with high-dose immunomodulating agents. These cytokines possess potent proinflammatory activities including stimulation of immune cell recruitment, proliferation, survival, differentiation, and effector functions (27, 28), and many of these cytokines (i.e., IL1a, IL1b, IL2, IFNα2, and IL12p70) have been implicated in inflammation that underlies autoimmune diseases (29, 30). Expression of these cytokines may help identify patients who are at risk of severe toxicity and who would therefore benefit from close monitoring for irAEs and early intervention or first-line treatment with anti-PD-1 monotherapy.

Cytokine expression in this study is reported as RFU rather than concentration. The low levels of cytokine expression in plasma meant that RFU signals were often below the standard protein curves used in the detection assays and this prevented absolute cytokine concentration calculations. However, we have previously shown that RFU are accurate indicators of relative cytokine levels (23), and RFU analysis provided more statistical power for testing differences in cytokine expression levels (24). Expression of the 65 cytokines in the cohorts was also measured across different assay plates and batches, which may have contributed to some discrepancies in RFU due to variations in assay lot (i.e., changes in assay reagents and antibodies) and machine calibration. However, cytokine data were normalized prior to analysis to correct for batch and plate variations as detailed in Materials and Methods.

The relationship between cytokine levels and patient response to immunotherapy was also examined. Although baseline expression of some cytokines was associated with treatment response and/or overall survival, these were not shared among the three cohorts. For instance, high levels of IL2 was only associated with improved survival and better treatment response in patients treated anti-PD-1 monotherapy. IL2 enhances $CD8^+$ T-cell cytolytic activity, and has been used clinically to augment T-cell responses in patients with metastatic melanoma (31–33). Although these results were not validated, these findings are provocative and warrant further investigation.

In conclusion, circulating cytokines are stable markers that predict severe immune-related toxicity in patients with melanoma treated with combination PD-1 and CTLA-4 inhibitors. These findings have important clinical implications in the prediction and early management of severe, potentially life-threatening immune-related toxicity, and should be validated further in larger, multi-institutional cohorts.

Disclosure of Potential Conflicts of Interest

A.M. Menzies is a consultant/advisory board member for Bristol-Myers Squibb, MSD, Novartis, Roche, and Pierre Fabre. A. Guminski is a consultant/advisory board member for Merck KgA, Bristol-Myers Squibb, Regeneron, Pfizer, Roche, and Sun Pharma. M.S. Carlino is a consultant/advisory board member for Bristol-Myers Squibb, MSD, Novartis, Amgen, and Pierre Fabre. R.F. Kefford is a consultant/advisory board member for Roche, Amgen, Bristol-Myers Squibb, Merck, Novartis, and TEVA, and reports receiving speakers bureau honoraria from Merck, Bristol-Myers Squibb, and Novartis. R.A. Scolyer is a consultant/advisory board member for

Table 3. ROC curve analysis using the sum of the 11-cytokine panel (CYTOX score)

	Cohort 2 (discovery)		Cohort 3 (validation)	
Geometric mean of 11 cytokines	**PRE**	**EDT**	**PRE**	**EDT**
AUC	0.7762	0.7651	0.6751	0.7003
95% CI	0.65–0.91	0.63–0.90	0.51–0.84	0.55–0.85
P value	0.0009	0.0014	0.0366	0.0168

Merck Sharp & Dohme, Novartis, NeraCare, and Myriad. G.V. Long is a consultant/advisory board member for Bristol-Myers Squibb, Novartis, Roche, Amgen, Pierre Fabre, Array, Merck, and Incyte. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.H. Lee, H. Rizos

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