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Personalized Medicine and Imaging

Clinical Cancer Research

Baseline Peripheral Blood Biomarkers Associated with Clinical Outcome of Advanced Melanoma Patients Treated with Ipilimumab

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Abstract

Purpose: To identify baseline peripheral blood biomarkers associated with clinical outcome following ipilimumab treatment in advanced melanoma patients.

Experimental Design: Frequencies of myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg), serum lactate dehydrogenase (LDH), routine blood counts, and clinical characteristics were assessed in 209 patients. Endpoints were overall survival (OS) and best overall response. Statistical calculations were done by Kaplan–Meier and Cox regression analysis, including calibration and discrimination by C-statistics.

Results: Low baseline LDH, absolute monocyte counts (AMC), $Lin^{-}CD14^{+}HLA\text{-}DR^{-/low}$-MDSC frequencies, and high absolute eosinophil counts (AEC), relative lymphocyte counts (RLC), and $CD4^{+}CD25^{+}FoxP3^{+}$-Treg frequencies were significantly associated with better survival, and were considered in a combination model. Patients (43.5%) presenting with the best biomarker signature had a 30% response rate and median survival of 16 months. In contrast, patients with the worst biomarkers (27.5%) had only a 3% response rate and median survival of 4 months. The occurrence of adverse events correlated with neither baseline biomarker signatures nor the clinical benefit of ipilimumab. In another model, limited to the routine parameters LDH, AMC, AEC, and RLC, the number of favorable factors (4 vs. 3 vs. 2–0) was also associated with OS ($P < 0.001$ for all pairwise comparisons) in the main study and additionally in an independent validation cohort.

Conclusions: A baseline signature of low LDH, AMC, and MDSCs as well as high AEC, Tregs, and RLC is associated with favorable outcome following ipilimumab. Prospective investigation of the predictive impact of these markers following ipilimumab and other treatments, e.g., PD-1 antibodies, is warranted. *Clin Cancer Res; 22(12); 2908–18. ©2016 AACR.*

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Introduction

Ipilimumab was the first agent to prolong survival of melanoma patients in randomized phase III studies (1, 2). However, only about 20% of treated patients experience a durable response, whereas all are at risk for side effects (3). The identification of patients who are most likely to experience clinical benefit will become increasingly important as alternative treatments such as combined targeted therapies, or anti-programmed cell death protein-1 (PD-1) antibodies, become available (4, 5).

Thus far, no reliable laboratory parameter is established in daily clinical routine predicting clinical outcome after ipilimumab treatment. Such biomarkers may be useful to select patients likely to benefit and vice versa to steer those with a low chance to alternative treatments. Moreover, biomarkers can shed light on the mechanisms of immune-mediated tumor rejection (6). Early studies with ipilimumab reported a correlation between favorable clinical outcome and the occurrence of autoimmunity after ipilimumab (7, 8). High serum lactate dehydrogenase (LDH) levels before, and increasing values during, treatment were reported to predict poor outcome (9–14). However, this marker is not regularly considered for treatment decisions in most countries.

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Translational Relevance

We report a prognostic combination model for melanoma patients treated with ipilimumab considering 6 baseline peripheral blood biomarkers. The spectrum comprised LDH as well as five immune cell populations, including $CD14^+HLA\text{-}DR^{-/low}$ MDSCs and $CD4^+CD25^+FoxP3^+$ Tregs. The observed negative impact of high MDSC frequencies translates into a strong rationale to investigate therapeutic strategies to deplete or inhibit these cells. Due to the complexity of flow cytometry, required for analysis of MDSCs and Tregs, we additionally defined a model limited to generally available routine markers. The resulting prognostic classification considering LDH, absolute monocyte and eosinophil counts, and relative lymphocyte counts delineates groups of patients with large differences in outcome. Our findings improve patient counseling and provide a rationale to investigate the predictive impact of these markers and the proposed combination in future studies not only for outcome after treatment with ipilimumab, but also at baseline for other treatments, such as using PD-1 antibodies.

Ipilimumab acts indirectly through immune cells by allowing T cell activation. $CD4^+$ T helper cells (15), $CD8^+$ cytotoxic T cells (16, 17), and those targeting melanoma-associated- (18) or neoantigens (19, 20) are in principle able to attack cancer cells and are most likely responsible for the beneficial effects of ipilimumab. Moreover, recent breakthroughs in immunotherapy, especially anti-PD-1 (5, 21) and anti-programmed cell death ligand-1 (PD-L1) antibodies (22), impressively demonstrate the capacity of a modulated immune system to reject cancer. Therefore, immune-related factors are promising biomarkers. Low serum concentrations of soluble CD25 (14) or C-reactive protein (CRP; ref. 23), and the presence of specific tumor mutations have been recorded in patients with favorable outcomes on ipilimumab treatment (19). The absolute lymphocyte count (ALC; refs. 11–13, 23, 24), the neutrophil count (25), or the neutrophil to lymphocyte ratio (26) was reported by different groups as other possible biomarkers.

Phenotypic characterization of immune cells provides detailed information about the patient's immune status (27). Populations with suppressive functions such as myeloid-derived suppressor cells (MDSC) or regulatory T cells (Tregs) are especially promising biomarker candidates because they might limit the supposed beneficial mode of action of ipilimumab (28). We recently demonstrated a strong prognostic relevance of MDSCs in melanoma patients (29). MDSCs have also been reported as predictive marker candidates for following ipilimumab administration (10, 30, 31).

The aim of the present study was to identify baseline peripheral blood biomarkers associated with overall survival (OS) and tumor response of melanoma patients treated with ipilimumab, by a comprehensive analysis of routine blood counts, frequencies of immune cell subsets analyzed by flow cytometry, and established prognostic factors (32). Moreover, we wanted to test whether the occurrence of adverse events (AE) after treatment with ipilimumab was associated with clinical outcome and/or baseline blood biomarkers.

Patients and Methods

Study design and patients

The study was conducted in two parts. The first part aimed to identify and confirm biomarker candidates, and to define prognostic models considering biomarker combinations. The second part aimed to validate the prognostic model based on routine markers as previously defined.

In the first part of the study, inclusion criteria were stage IV melanoma, treatment with at least one dose of ipilimumab at 3 or 10 mg/kg in the metastatic (not adjuvant) setting, and availability of cryopreserved baseline peripheral blood mononuclear cells (PBMC). Patients with uveal or mucosal melanoma were excluded. All patients gave written informed consent for biobanking, and use of biomaterials and clinical data for scientific purposes. This part was approved by the Ethics Committee, University of Tuebingen (approval 524/2012B02).

In the first part of the study, two separate cohorts of patients (identification and confirmation cohort) were analyzed. The identification cohort comprised 105 patients from Amsterdam, Essen, Lausanne, Nantes, and Tuebingen. The remaining 104 patients from Naples, New York, and Siena were aligned to the confirmation cohort aiming at a balanced sample size of both cohorts. Differences in OS according to 28 factors were investigated in the identification cohort. These factors were gender, age, and the pattern of visceral tumor involvement (soft-tissue and/or lung-only vs. involvement of other organs), the presence of brain metastases, LDH, absolute leucocyte counts, absolute and relative lymphocyte-, monocyte- and eosinophil counts, and the frequencies of 16 immune cell populations analyzed by flow cytometry (Supplementary Table S1 and S2). LDH was analyzed by means of the LDH ratio [actual value divided by the upper limit of normal (ULN)]. All blood parameters derived from blood draws taken within 28 days before the first dose.

The analysis of the identification cohort aimed to identify biomarker candidates. Candidates and respective cutoff points for continuous variables were defined by applying an optimization algorithm similar to those published earlier (10, 33). In detail, differences in OS for continuous variables were analyzed using a modified approach of maximally selected *P* values based on log-rank tests at different cutoff points to divide the identification cohort for each factor into two or three groups. First, only central cutoff points were analyzed resulting in two balanced groups. A central cutoff point was considered for survival analysis if the resulting smaller group comprised at least 25% of all patients. Of all analyzed cutoff points, the lowest significant log-rank *P* value was chosen as cutoff candidate 1. If no significant log-rank *P* value was observed for any analyzed central cutoff, potential eccentric cutoffs (the resulting smaller group comprised at least 10% of patients) were analyzed. Of all analyzed eccentric cutoff points, the lowest significant log-rank *P* value was chosen as cutoff 1. For continuous variables with an established cutoff 1, the definition of a second cutoff point resulting in three groups according to this variable was attempted. A central second cutoff point was considered for survival analysis, if the smallest of the resulting three groups comprised at least 25% of discovery cohort patients. Differences in OS between the three groups were analyzed using pairwise comparison and only cutoff points resulting in significant differences for each group combination were further considered. Of those, the cutoff point resulting in the lowest significant log-rank *P* value was chosen as cutoff 2. If no central

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Table 1. Patient and treatment characteristics

Factor	Category	Identification cohort ($n = 105$) *n* (%)	Confirmation cohort ($n = 104$) *n* (%)	Identification and confirmation cohort combined ($n = 209$) *n* (%)	Validation cohort ($n = 406$) *n* (%)
Clinical site	Amsterdam	54 (51.4)		54 (25.8)	94 (23.2)
	Essen	15 (14.3)		15 (7.2)	19 (4.7)
	Heidelberg				113 (27.8)
	Lausanne	10 (9.5)		10 (4.8)	
	Nantes	10 (9.5)		10 (4.8)	49 (12.1)
	Naples		20 (19.2)	20 (9.6)	34 (8.4)
	New York		49 (47.1)	49 (23.4)	
	Siena		35 (33.7)	35 (16.7)	38 (9.4)
	Tuebingen	16 (15.2)		16 (7.7)	59 (14.5)
Gender	Male	55 (52.4)	63 (60.6)	118 (56.5)	192 (47.3)
	Female	50 (47.6)	41 (39.4)	91 (43.5)	214 (52.7)
Age	≤50 years	39 (37.1)	28 (26.9)	67 (32.1)	119 (29.3)
	>50 years	23 (21.9)	26 (25.0)	49 (23.4)	86 (21.2)
	>60 years	22 (21.0)	25 (24.0)	47 (22.5)	121 (29.8)
	>70 years	21 (20.0)	25 (24.0)	46 (22.0)	80 (19.7)
	Median age	54	60	58	60
M category (AJCC)	M1a	11 (10.5)	9 (8.7)	20 (9.6)	26 (6.4)
	M1b	14 (13.3)	15 (14.4)	29 (13.9)	43 (10.6)
	M1c	78 (74.3)	80 (76.9)	158 (75.6)	336 (82.8)
	Unknown	2 (1.9)		2 (1.0)	1 (0.2)
Visceral involvement	Soft tissue only	14 (13.3)	13 (12.5)	27 (12.9)	41 (10.1)
	Lung	15 (14.3)	30 (28.8)	45 (21.5)	56 (13.8)
	Other organs	76 (72.4)	61 (58.7)	137 (65.6)	308 (75.9)
	Unknown				1 (0.2)
LDH	Elevated	45 (42.9)	51 (49.0)	96 (45.9)	184 (45.3)
	Normal	56 (53.3)	53 (51.0)	109 (52.2)	222 (54.7)
	Unknown	4 (3.8)		4 (1.9)	
Treatment background	CA-184-128 (3 mg/kg, local IL-2)	14 (13.3)		14 (6.7)	
	CA-184-169 (3 or 10 mg/kg)	5 (4.8)		5 (2.4)	
	Early access program (3 mg/kg)	34 (32.4)	63 (60.6)	97 (46.4)	117 (28.8)
	Regular prescription (3 mg/kg)	52 (49.5)	39 (37.5)	91 (43.5)	289 (71.2)
	BMS-024 (10 mg/kg, dacarbazine)		2 (1.9)	2 (1.0)	
Doses applied	1	9 (8.6)	2 (1.9)	11 (5.3)	23 (5.7)
	2	13 (12.4)	4 (3.8)	17 (8.1)	41 (10.1)
	3	16 (15.2)	16 (15.4)	32 (15.3)	43 (10.6)
	4	67 (63.8)	82 (78.8)	149 (71.3)	296 (72.8)
Best clinical response (irRC)	Complete response	3 (2.9)	4 (3.8)	7 (3.3)	
	Partial response	17 (16.2)	13 (12.5)	30 (14.4)	
	Stable disease	15 (14.3)	14 (13.5)	29 (13.9)	
	Progressive disease	69 (65.7)	63 (60.6)	132 (63.2)	
	Unknown	1 (1.0)	10 (9.6)	11 (5.3)	406 (100)

Abbreviations: AJCC, American Joint Committee on Cancer; IL-2, interleukin-2; irRC, immune-related response criteria; LDH, lactate dehydrogenase.

second cutoff point could be established, potential eccentric second cutoff points were considered for survival analysis, if the smallest of the resulting three groups comprised at least 10% of patients. Differences in OS between the three groups were analyzed using pairwise comparisons, and only cutoff points resulting in significant differences for each group-combination were further considered. Of those, the cutoff point resulting in the lowest significant log-rank *P* value was chosen as cutoff 2.

Factors that were not significantly correlated with OS in the identification cohort were not further considered. Factors categorizing patients into groups with significant differences in OS, as defined in the identification cohort, were subsequently tested for their association with OS in the confirmation cohort. Clinical responses were assessed by the investigators of the respective clinical site and categorized as either complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) according to immune-related response criteria (irRC; ref. 34). A blinded or independent radiologic review was not conducted. The best overall response (bOR) was defined by the best achieved response between starting administration of ipilimumab and progression or start of a new systemic treatment considering all available tumor assessments in this time period. Patients were classified as having experienced a clinical response if the bOR was PR or CR and clinical benefit in case of SD, PR, or CR. Data on grade III, IV, and V AEs according to common toxicity criteria, which were at least possibly related to ipilimumab, were collected for patients of the identification and confirmation cohort. Colitis/diarrhea, dermatitis, hypophysitis, hepatitis, and the development of Guillain–Barré syndrome were classified as immune-related AEs (irAE).

After completion of this first part, a validation study was conducted in 406 patients from seven clinical sites (Ethics approval 234/2015B02). In contrast to the first part, only patients treated at 3 mg/kg were considered. The collected data

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were limited to routine blood counts, LDH, and clinical parameters. PBMCs were not available for flow cytometric analysis. OS served as endpoint.

Flow cytometry

PBMCs were thawed and immediately analyzed by flow cytometry. F_c receptors were blocked with human IgG (Gamunex; Talecris), and dead cells were excluded by ethidium monoazide labeling (EMA, Biotinum). Staining was performed separately for the analysis of myeloid cells and T-cells/Tregs using antibody panels described in detail in Supplementary Table S1. Data were acquired with a BD LSR-II with FACS-Diva software V6.1.3 (BD) and analyzed with FlowJo V9.3.2 (Tree Star). Gating strategies are displayed in Supplementary Fig. S1.

Statistical analysis

OS time was defined from the date of the first dose of ipilimumab to the date of last follow-up or death. Disease-specific survival probabilities were estimated according to the Kaplan–Meier method, and compared using log-rank tests. Only deaths due to melanoma were considered; other causes of death were regarded as censored events. Cox proportional hazard regression models were applied to determine the impact of confirmed single factors. Results of Cox regression analysis are described by means of hazard ratios (HR), and *P* values (Wald test). Patients with missing data in variables analyzed in the given model were excluded. The concordance index (c-index) was calculated for different models as a measure of the discriminatory ability that allows comparison of models. A model with a c-index of 0.5 has no predictive value, a model with a c-index of 1 would allow a perfect prediction of the patient's outcome (35). The concordance index was analyzed using the survConcordance function in the survival package for R. Calibration of the combination models was calculated using the calibrate function in the rms package of R and the Kolmogorov–Smirnov test for survival data using the coxph function in the survival package of R. Associations between clinical response and biomarker categories were analyzed by χ^2 and Fisher exact tests. Throughout the analysis, $P < 0.05$ were considered statistically significant. Analyses were carried out using SPSS 22 (IBM) and R 3.2.1 (R Foundation for Statistical Computing).

Results

Patients and treatments

A total of 209 patients treated with ipilimumab at eight clinical sites were included in the first part of the study. A detailed listing of patient and treatment characteristics is presented in Table 1. Median age was 58 years, and 56.5% were male. One hundred fifty-eight individuals were assigned to the M category M1c (76.3%), 29 to M1b (14%), and 20 to M1a (9.7%). Treatment was mainly administered in the compassionate use program (46.4%) or after marketing approval (43.5%). Two hundred six patients received at least one prior systemic treatment before ipilimumab. Of 198 with available data on the bOR 37 (18.7%) experienced a CR or PR. An additional 29 patients had SD, resulting in a clinical benefit rate of 33.3%. One hundred sixty deaths were observed during follow-up (159 were melanoma-related, one was due to sepsis). Median OS after start of treatment was 7 months. Median follow-up was 19 months for patients who were alive at the last follow-up, and 5 months for those who died (Table 1).

Validation was subsequently performed in the second part of the study in an additional independent cohort of 406 patients. Those patients were treated in the compassionate use program ($n = 117$; 28.8%) or after marketing approval ($n = 289$; 71.2%). Seventy-seven patients (19%) received ipilimumab as a first-line treatment, while the remaining patients had at least one prior systemic treatment. Among patients treated with ipilimumab included in the validation cohort the median age was 60 years, 47% were male. Of 405 individuals 336 were assigned to the M-category M1c (83%), 43 to M1b (10.6%), and 26 to M1a (6.4%). The M category was unknown in 1 patient. LDH was elevated in 184 (45.3%). Two hundred ninety-six patients received all 4 doses, while in the remaining patients, treatment was stopped after 1 to 3 doses. Median follow-up was 15 months for patients who were alive at the last follow-up, and 7 months for those who died. Median OS after the start of ipilimumab was 8 months (Table 1).

Identification and confirmation of biomarkers

Altogether, 28 variables were investigated in 105 patients (identification cohort) to identify biomarker candidates. Of these, 8 were not associated with prognosis, including the presence of brain metastases. Thirteen variables were associated with OS at one, and 7 at two, optimized cutoff points. In total, 27 variable/cutoff combinations derived from 20 biomarkers were identified as candidates and further assessed in 104 patients (confirmation cohort). Here, 6 variables were also significantly associated with OS at one, and 2 variables at two previously defined cutoff points. In total, 10 biomarker/cutoff combinations derived from 8 biomarkers were confirmed and further considered. All variables, and survival analyses according to the cohorts and variable/cutoff combinations, are presented in Supplementary Table S2.

Survival analysis using confirmed biomarkers

OS according to eight confirmed biomarkers (LDH and $Lin^-CD14^+HLA\text{-}DR^{-/low}$ MDSCs at two cutoff points = 10 biomarker/cutoff combinations) in all patients of the combined identification and confirmation cohorts is presented in Table 2. LDH was the strongest biomarker for classifying patients according to OS into three groups. Median OS was 10 months for patients with baseline LDH up to 1.2-fold higher than the ULN, but for those with >1.2-fold or >2.3-fold, it was only 5 and 2 months, respectively ($P = 1.54 \times 10^{-12}$; Fig. 1A). A relative lymphocyte count (RLC) <10.5% identified patients with a 1-year survival probability of only 5% ($P = 3.30 \times 10^{-12}$; Fig. 1B). However, a low frequency of $Lin^-CD14^+HLA\text{-}DR^{-/low}$ MDSCs was associated with the highest probability of long-term survival. Thus, 2-year survival probability after ipilimumab initiation was 34.5% for 99 patients with MDSC frequencies <5.1%, while there were no survivors among 65 patients with higher baseline levels ($P = 6.73 \times 10^{-11}$; Fig. 1C). An absolute monocyte count (AMC) <650/μL (Fig. 1D) and a frequency of $CD14^+$ monocytes <28% were also strongly associated with favorable outcome ($P = 1.35 \times 10^{-08}$ and 6.58×10^{-07}, respectively). Additionally, absolute (Fig. 1E) and relative eosinophil counts (AEC and REC) were positively correlated with survival ($P = 5.06 \times 10^{-05}$ and 2.14×10^{-04}, respectively). Baseline frequencies of $CD4^+CD25^+FoxP3^+$ Tregs ≥1.5% were associated with good prognosis after initiation of ipilimumab ($P = 8.70 \times 10^{-05}$; Fig. 1F).

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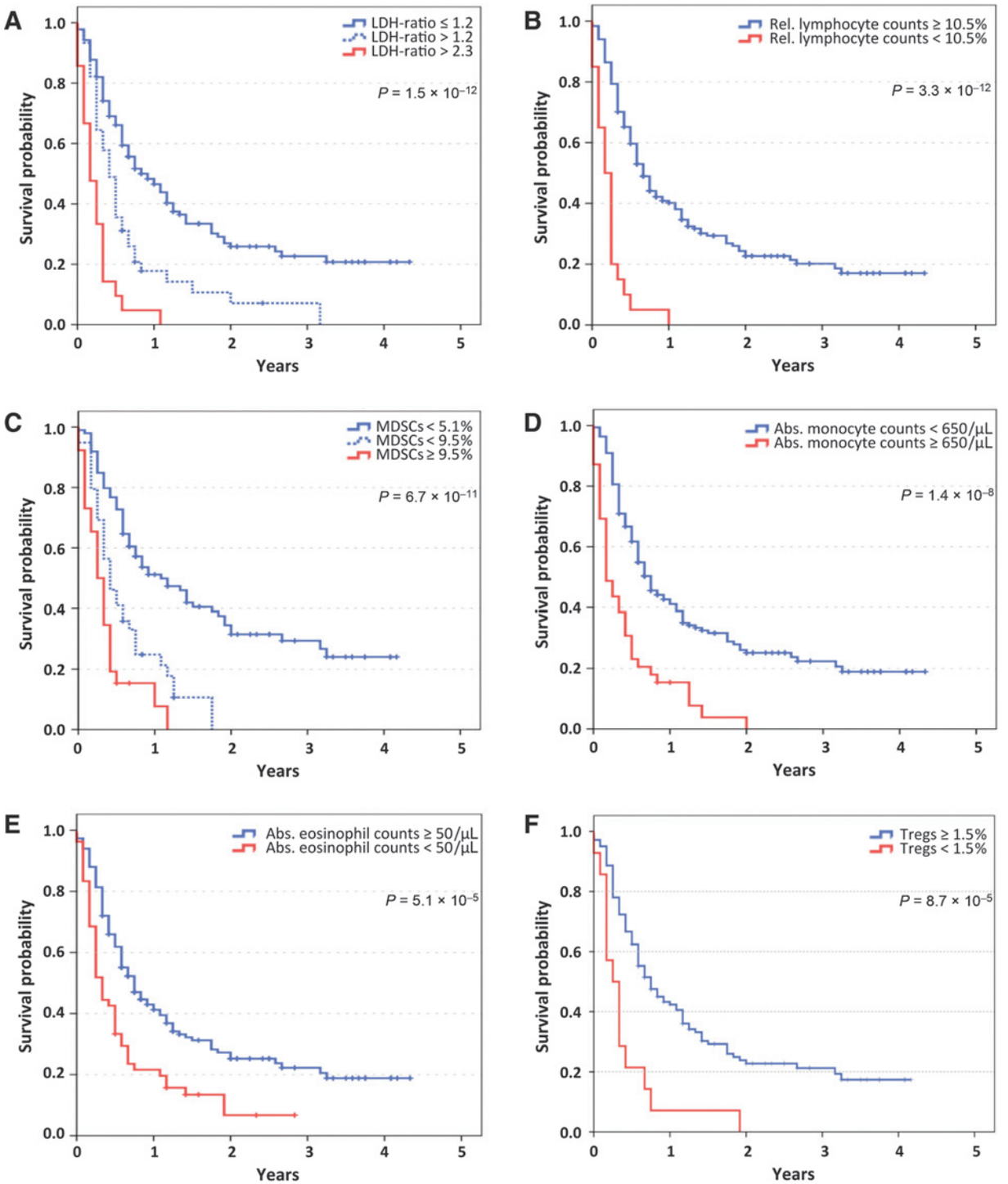

Figure 1.

OS according to confirmed biomarkers. Kaplan–Meier analysis of OS in the identification and confirmation cohort ($n = 209$) according to LDH ratio (the measured LDH serum concentration divided by the upper limit of normal; A), RLC (B), frequency of $Lin^{-}CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs (C), AMC (D), AEC (E), and frequency of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs (F). Censoring is indicated by vertical lines; *P* values were calculated by log-rank statistics.

Definition of a combination model

Cox regression analysis was performed to determine the relative impact of confirmed biomarkers. LDH (at both cutoff points), MDSCs, RLC, AMC, and AEC (each at one cutoff) remained in the model as significantly independent biomarkers. REC, Tregs, or $CD14^{+}$ monocyte frequencies did not

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Table 2. OS according to confirmed biomarkers

					Univariate survival analysis							
					Median survival	1-Year survival		2-Year survival		3-Year survival		
Factor	Total n	Categories	*n* (%)	% Dead	(months)	rate (95% CI)		rate (95% CI)		rate (95% CI)		*P*
LDH-ratio	205	≤1.2	139 (67.8)	69.1	10	48.3	(39.9–56.7)	27.0	(18.8–35.2)	22.6	(14.4–30.8)	1.54E−12
		>1.2	44 (21.5)	88.6	5	18.2	(6.2–30.2)	10.9	(0.3–21.5)	7.3	(0.0–16.5)	
		>2.3	22 (10.7)	100.0	2	4.5	(0.0–13.1)					
RLC	204	<10.5%	20 (9.8)	100.0	2	5.0	(0.0–14.6)					3.30E−12
		>10.5%	184 (90.2)	72.8	8	40.8	(33.6–48.1)	24.3	(17.4–31.3)	20.1	(13.2–27.0)	
AMC	204	<650/μL	165 (80.9)	70.9	9	42.6	(34.9–50.4)	26.1	(18.6–33.5)	22.3	(14.8–29.9)	1.35E−08
		>650/μL	39 (19.1)	94.9	2	15.4	(4.1–26.7)	3.8	(0.0–11.0)			
AEC	204	<50/μL	54 (26.5)	88.9	4	21.6	(10.5–32.7)	6.7	(0.0–14.8)			5.06E−05
		>50/μL	150 (73.5)	70.7	9	42.9	(34.8–51.1)	27.2	(19.3–35.1)	22.2	(14.3–30.1)	
REC	204	<1.5%	89 (43.6)	85.4	6	24.8	(15.5–34.1)	12.1	(4.2–20.0)	7.5	(0.5–14.6)	2.14E−04
		>1.5%	115 (56.4)	67.8	9	46.8	(37.5–56.1)	29.2	(20.0–38.4)	25.9	(16.7–35.2)	
$CD4^+CD25^+FoxP3^+$ Tregs	155	<1.5%	14 (9.0)	100.0	3	7.1	(0.0–20.6)					8.70E−05
		>1.5%	141 (91.0)	72.3	9	43.3	(34.9–51.7)	23.8	(15.9–31.8)	21.2	(13.4–29.1)	
$CD14^+$ Monocytes	189	<28%	162 (85.7)	70.4	9	43.5	(35.7–51.4)	26.4	(18.8–34.0)	22.9	(15.3–30.5)	6.58E−07
		>28%	27 (14.3)	96.3	4	13.3	(0.0–26.7)					
$Lin^-CD14^+HLA\text{-}DR^{-/low}$ MDSCs	164	<5.1%	99 (60.4)	64.6	13	51.2	(41.1–61.3)	34.5	(24.2–44.9)	29.4	(19.0–39.8)	6.73E−11
		≥5.1%	39 (23.8)	87.2	5	24.9	(11.1–38.6)					
		≥9.5%	26 (15.9)	92.3	3	15.4	(1.5–29.3)					

Abbreviations: AEC, absolute eosinophil counts; AMC, absolute monocyte counts; HR, hazard ratio; LDH, lactate dehydrogenase; MDSCs, myeloid-derived suppressor cells; REC, relative eosinophil counts; RLC, relative lymphocyte counts; Tregs, regulatory T cells.

add further significant independent prognostic information (Table 3, left).

Next, the discriminatory ability of the initial model considering the relative impact of all 5 independent biomarkers in combination and 13 alternative combination models was analyzed using C-statistics. The best discriminatory ability (Supplementary Fig. S2A and S2B) and satisfactory calibration (Supplementary Fig. S3A) was achieved when Tregs were likewise considered in addition to LDH (at both cutoff points), MDSCs, RLC, AMC, and AEC in the combination model (c-index = 0.712), despite this factor having no significant independent impact according to Cox regression analysis (Table 3, middle). The latter model combining 6 biomarkers (LDH at two cutoff points), including Tregs, was selected for further analysis (combination model 1).

Table 3. Multivariate models

	Multivariate analysis of significantly independent factors ($n = 138$)			Multivariate analysis including Tregs (combination model 1) ($n = 138$)			Combination model 2 considering LDH (elevated vs. normal) and blood count parameters[a] only ($n = 200$)		
Factor	Category	HR	*P*	Category	HR	*P*	Category	HR	*P*
LDH ratio	>2.3	4.9	0.0156	>2.3	5.2	0.0103	Elevated	1.9	0.0003
	>1.2	1.8	0.0263	>1.2	1.8	0.0336	Normal		
	<1.2	1.0		<1.2	1.0			1.0	
RLC	<10.5%	2.4	0.0110	<10.5%	2.6	0.0071	<10.5%	4.2	<0.0001
	>10.5%	1.0		>10.5%	1.0		>10.5%	1.0	
AMC	≥650/μL	2.0	0.0171	≥650/μL	2.0	0.0218	≥650/μL	2.2	0.0001
	<650/μL	1.0		<650/μL	1.0		<650/μL	1.0	
AEC	<50/μL	1.7	0.0225	<50/μL	1.6	0.0285	<50/μL	1.7	0.003
	>50/μL	1.0		>50/μL	1.0		>50/μL	1.0	
REC	<1.5%	Not independent		<1.5%	Not considered		<1.5%	Not independent	
	>1.5%			>1.5%			>1.5%		
$Lin^-CD14^+HLA\text{-}DR^{-/low}$ MDSCs	≥9.5%	Not independent		≥9.5%	Not considered		Not considered		
	≥5.1%	2.6	<0.0001	≥5.1%	2.5	0.0001			
	<5.1%	1.0		<5.1%	1.0				
$CD4^+CD25^+FoxP3^+$ Tregs	<1.5%	Not independent		<1.5%	1.8	0.1439	Not considered		
	>1.5%			>1.5%	1.0				
$CD14^+$ monocytes	<28%	Not independent		<28%	Not considered		Not considered		
	≥28%			≥28%					

Abbreviations: AEC, absolute eosinophil counts; AMC, absolute monocyte counts; HR, hazard ratio; LDH, lactate dehydrogenase; MDSCs, myeloid-derived suppressor cells; REC, relative eosinophil counts; RLC, relative lymphocyte counts; Tregs, regulatory T cells hazard ratio.
[a]Relative lymphocyte count, AMC, AEC, and REC.

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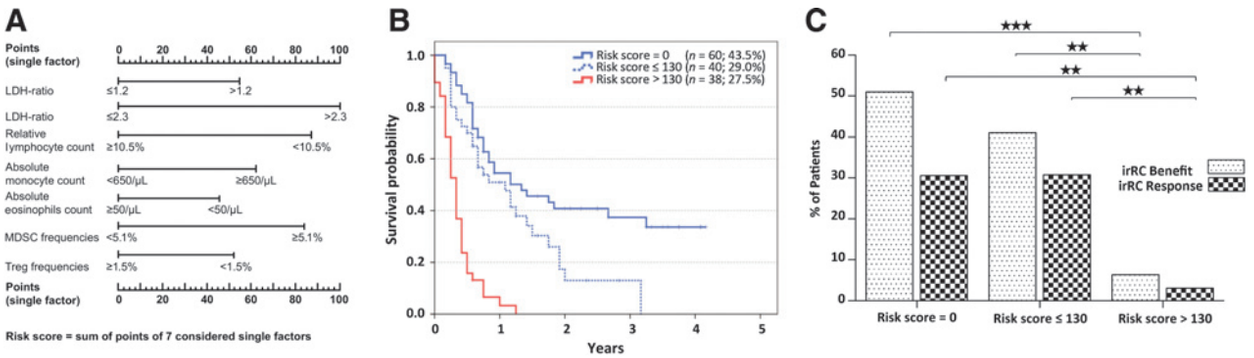

Figure 2.
OS and tumor response according to combination model 1. A nomogram-based linear predictor measure was calculated for each patient considering the relative impact of single factors according to Cox regression analysis (A). In combination model 1, the LDH ratio (at two cutoff points), the absolute eosinophil and monocyte counts, the relative lymphocyte count, the frequency of $Lin^{-}CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs and $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs were considered. Kaplan–Meier analysis of OS is presented according to the patient's individual risk score, which was calculated as the sum of the values of 7 separate factors. Censoring is indicated by vertical lines (B). The best overall tumor response according to irRC was analyzed either as the rate of patients with irRC benefit (sum of those with complete responses, partial responses, and stable disease) or irRC response (sum of those with complete or partial responses; C). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Classification of patients in this model was based on a linear predictor score (risk score) accounting for the relative impact of each marker in the combination model (Fig. 2A).

The 2-year survival rate for patients with favorable values for all 6 biomarkers (risk score = 0) was 40.8% compared with 17.3% for those with risk scores ≤130. In contrast, none of the patients with risk scores >130 survived longer than 15 months (Fig. 2B). Moreover, the rate of clinical responses differed strongly between risk score groups (Fig. 2C). The response rate in patients with risk scores of 0, ≤130, or >130 was 31%, 31%, and 3% (51%, 41%, and 6% rate of clinical benefit, respectively) according to irRC.

Definition of a combination model limited to routine markers

Next, we developed a less complex model that allows immediate application in daily clinical practice. Therefore, we focused exclusively on the impact of clinical parameters and factors available in the routine laboratory setting. Factors requiring low cytometry, for example the determination of subpopulations of MDSCs and Tregs, were not considered as this technique is not broadly available and the exact determination of these immune parameters is not yet standardized. In contrast to model 1, we aimed to avoid the need for calculations here. Therefore, the number of favorable factors in combination model 2 was counted instead of calculating the risk score for the individual patient (model 1). Moreover, LDH was categorized as elevated versus normal, instead of considering the LDH ratio. According to Cox regression analysis, an RLC < 10.5% appeared to be the strongest independent factor (HR, 4.2; $P < 0.0001$) followed by an AMC ≥650/μL (HR, 2.2; $P = 0.0001$), elevated LDH (HR, 1.9; $P = 0.0003$), and a low AEC < 50/μL (HR, 1.7; $P = 0.003$). The REC did not add independent power (Table 3, right). The count of values classified as favorable for all 4 independent factors was selected as outcome measure of combination model 2. This model was chosen based on the highest discriminatory ability (c-index = 0.690; Supplementary Fig. S2B) of all possible combination models considering the five routine markers (Supplementary Fig. S2C and S2D) and satisfactory calibration (Supplementary Fig. S3B). The 2-year survival probability of patients with favorable

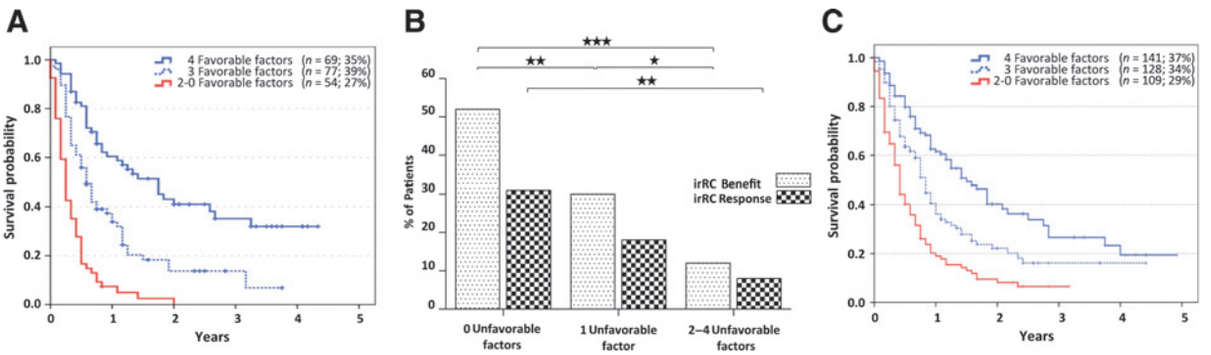

Figure 3.
OS and tumor response according to combination model 2. In combination model 2, only routine biomarkers, available in daily practice, were considered. In addition to the absolute eosinophil and monocyte counts, the relative lymphocyte counts and LDH (categorized as elevated vs. normal) were integrated. Patients were stratified according to the number of favorable factors for Kaplan–Meier analysis of OS. Censoring is indicated by vertical lines (A). The best overall tumor response according to irRC was analyzed either as the rate of patients with irRC benefit (sum of those with complete responses, partial responses and stable disease) or irRC response (sum of those with complete or partial responses; B). The association with OS of combination model 2 was confirmed in an independent validation cohort of 378 patients with available data for all 4 factors (C). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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profiles for all 4 markers was 43.1% compared with 13.7% for those with one, and 2.5% for those with two or more unfavorable values ($P < 0.001$ for all pairwise comparisons of categories; Fig. 3A). Similar to the first model, there was a strong correlation with the bOR (Fig. 3B). The response rate in patients with 4, 3, and 2–0 favorable baseline biomarker results was 31%, 18%, and 8% (52%, 30%, and 12% rate of clinical benefit, respectively) according to irRC.

Validation of the combination model limited to routine markers

Finally, the factors considered in combination model 2 were additionally analyzed in an independent cohort of 406 patients treated with ipilimumab. All 4 single baseline factors (LDH elevated vs. normal, RLC < vs. ≥ 10.5%, AMC < vs. ≥ 650/μL, AEC < vs. ≥ 50/μL) were significantly associated with OS in univariate analysis of the validation cohort (all log rank $P < 0.05$). Large differences in OS were again observed according to the number of favorable baseline factors for patients treated with ipilimumab ($P < 0.001$ for all pairwise comparisons of categories 4 vs. 3 vs. 2–0 favorable factors; Fig. 3C) and the c-index was 0.652. The 2-year survival probability of patients with favorable profiles for all 4 markers was 40.2% compared with 22.1% for those with one, and 9.5% for those with two or more unfavorable values.

Correlations with grade III/IV/V AEs

AEs of grade III or higher were reported for 26 (12.6% of 207 evaluable patients) and irAE in 23 patients (11.1%). Colitis/diarrhea was most frequently observed ($n = 11$; 5.3%). Less frequent AEs were dermatitis ($n = 5$; 2.4%), hypophysitis, and hepatitis (each $n = 3$; 1.4%). The occurrence of nausea, headache/asthenia, neutropenia, orthostatic dysregulation, and the development of Guillain–Barré syndrome was noted in 1 patient, respectively. The severity of all AEs was classified as grade III, and no grade IV or V toxicities were reported. The occurrence of AEs was neither correlated with OS since starting ipilimumab, nor with best clinical response, nor with the combination groups of baseline biomarkers (Supplementary Fig. S4).

Further characterization of the proposed combination models

Seven patients of the identification and the confirmation cohorts received either 10 mg/kg ipilimumab or were treated at 3 or 10 mg/kg in a blinded manner. As the applied dose may confound the biomarker results, an additional analysis was conducted excluding those patients. All independent factors considered in the models as described in Table 3 had also significant independent impact in the reduced cohort of patients treated at 3 mg/kg ipilimumab ($n = 202$). HRs changed only marginally (Supplementary Table S3).

Moreover, confounding effects of subsequent therapies were analyzed in 71 patients from the identification and confirmation cohorts who had received at least one systemic treatment after ipilimumab. They were treated with BRAF/MEK inhibitors ($n = 24$), PD-1/PD-L1 antibodies ($n = 28$), or chemotherapy/other treatments ($n = 33$). Patients receiving PD-1/PD-L1 antibodies had an exceptionally long OS (Supplementary Fig. S5B), and were overrepresented in the prognostically favorable biomarker groups (Supplementary Fig. S5A). However, the prognostic impact of both biomarker combination models remained significant ($P < 0.018$ or less for all pairwise comparisons of categories of the respective model), if patients treated with PD-1/PD-L1 antibodies were excluded (Supplementary Fig. S5C and S5D).

Discussion

In the current study, the LDH ratio, AMC, AEC, RLC, and the frequency of MDSCs and Tregs were found to represent baseline peripheral blood biomarkers affecting OS of melanoma patients treated with ipilimumab. The LDH ratio was a strong baseline biomarker associated with prognosis, as similarly reported by others (10–13). We did not observe differences in OS according to the baseline ALC (11). However, a low AEC correlated with favorable outcome. Similar findings were reported by Schindler and colleagues at the ASCO meeting 2013 (36) and an increase of eosinophils during ipilimumab was associated with OS in the study of Delyon and colleagues (12). Our study is the first to report a negative impact of high AMC, consistent with a similar association with the frequency of $CD14^+$ monocytes analyzed by flow cytometry. An association of high AMC with poor prognosis was reported before (37, 38), but baseline counts were not predictive for ipilimumab-treated patients in the study of Kitano and colleagues (10). However, a different cutoff point used to categorize patients (300/μL vs. 650/μL in our study) may explain the divergent results. A low baseline frequency of $Lin^-CD14^+HLA\text{-}DR^{-/low}$ MDSCs was a powerful indicator of benefit and was the strongest stand-alone factor of the entire study to indicate long-term survival. Similar results were previously reported from two single-center studies (10, 30) and a recent study of Gebhardt and colleagues (31). The inverse correlation of MDSC frequencies and OS following ipilimumab and the prognostic relevance for melanoma patients with distant metastasis in general (29) provide a rationale to pursue therapeutic strategies aiming at depleting these cells. Blockade of the suppressive function of MDSCs using cyclooxygenase-2 (COX-2)/prostaglandin E2 pathway inhibitors (39, 40) or phosphodiesterase inhibitors (41) represents other possible approaches, which may be tested as monotherapies or in combination with ipilimumab.

Interestingly, higher baseline frequencies of circulating $CD4^+CD25^+FoxP3^+$ Tregs were associated with improved OS. Tregs represent direct target cells of ipilimumab due to their constitutive CTLA-4 expression. Therefore, a high baseline frequency might render patients more susceptible to anti–CTLA-4 antibodies. This hypothesis is strongly supported by the observed correlation between decreasing levels of circulating Tregs during ipilimumab and favorable outcome (9). However, conflicting results have also been reported (42).

The T cell response, which is crucial for immunological melanoma rejection in patients treated with ipilimumab (16, 17, 19, 20), is balanced by interactions between T cells and regulatory cells (28). All five cellular compartments, which we found to associate with outcome upon ipilimumab treatment (eosinophils, lymphocytes, monocytes, Tregs and MDSCs), are involved in this complex regulatory network. For instance, eosinophils have important functions for tumor surveillance and were described as potent effectors for tumor rejection in mouse models (43, 44). MDSCs and Tregs have been shown to exert suppressive function on T cells, thereby possibly counteracting the beneficial effect of ipilimumab (28, 45).

We propose a combination model for outcome of ipilimumab treatment defined by six baseline biomarkers. Based on the LDH ratio, the AMC and AEC, the RLC and the frequency of MDSCs and

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Tregs, patients were classified into three groups with clinically meaningful differences in survival and response rate. Additionally, we propose a biomarker signature that could be easily implemented in routine clinical settings. This simplified classification based on LDH, AMC, and AEC, and RLC allowed identification of 27% of all patients with a median survival of 3 months, no survivors beyond 2 years, and a response rate of only 8%. In contrast, this combination model also identified 35% of all patients presenting favorable values for all four biomarkers with a 35% probability of surviving longer than 3 years and response rates of ∼30%. In cases where several treatment options may be available for the individual patient, these findings may affect treatment selection and sequence. Of note, based on the discriminatory abilities, both models were superior for prognosis prediction than considering LDH alone. The respective c-indices were 0.712 and 0.690 for combination models 1 and 2, in contrast to 0.617 for the LDH ratio categorized as >2.3 vs. >1.2 vs. ≤1.2, or 0.598 if LDH was categorized as elevated versus normal in the combined identification and confirmation cohorts. OS was similar for the poorest prognostic group according to model 1 (risk score >130) or model 2 (0–2 favorable factors) compared with patients with LDH ratio >2.3. However, the latter was only true for 10.7% of patients, while the usage of combination model 1 or model 2 allowed the identification of 27.5% and 27.0% of patients with poorest prognosis. Another advantage of combination model 1 compared with the consideration of LDH alone is the identification of long-term survivors. Three years after the start of ipilimumab, OS was 37.4% and 35.2% among patients with risk score = 0 (model 1) or 4 of 4 favorable factors (model 2), in contrast to only 22.6% or 25.1% for patients with LDH ratio ≤1.2 or normal LDH, respectively. A model based on the number of involved organs, the Eastern Cooperative Oncology Group (ECOG) performance status, and LDH prior to initiation of ipilimumab treatment was recently reported by Diem and colleagues (46). In contrast to our study, OS was longer, and response rates were higher in the best prognostic category, but this group comprised only 13% of patients (∼35% in our model 2). The combined consideration of the clinical factors as described by Diem and colleagues, together with the peripheral blood factors as presented here, might further improve the prognostic modeling for patients receiving ipilimumab in the future. Importantly, in this study we followed REMARK recommendations (47) and confirmed the association between 10 variable/cutoff combinations and OS in a confirmation cohort. Altogether, 209 patients from eight clinical sites and six different countries were included, minimizing the risk that our results are confounded by patient selection, regional- or site-specific influences. Nevertheless, there are limitations to our study that need to be considered. Other factors, for example the ECOG performance status or prior treatments, for example with BRAF/MEK inhibitors, may affect outcome following ipilimumab or the biomarker results, which were not analyzed in detail here. The results of factors analyzed by flow cytometry may be confounded by varying site-specific protocols for isolation, freezing, or storage of PBMC and might not reflect the actual immune milieu *in vivo*, for example due to differences in susceptibility to cryopreservation between immune cell populations (48). We were able to validate the prognostic relevance of the combination model limited to routine factors in an additional independent cohort of 406 patients. The number of favorable factors (4 vs. 3 vs. 2–0) according to this model again was strongly associated with OS ($P < 0.001$ for all pairwise comparisons) in patients of the validation cohort, although the discriminatory ability was lower than that in the main study (c-indices 0.652 vs. 0.690). Thus, further validation is warranted. This is particularly important because patients analyzed here were heterogeneous regarding the treatment background. Patients were treated either after marketing approval, in the compassionate use program or in different clinical trials. Site-specific treatment procedures and patient selection guidelines or the inclusion/exclusion criteria in the clinical trials may lead to a selection bias and confounding effects on the biomarker results. The question whether the suggested signatures are prognostic in general or specifically predictive for outcome after ipilimumab cannot be answered by our study. This key question needs to be addressed in future studies, including patients in other clinical situations; e.g., tumor-free individuals in earlier stages after surgery, or prior to other treatments; e.g., with PD-1 antibodies or in the context of randomized controlled clinical trials.

Early clinical studies reported a correlation between the occurrence of autoimmunity after ipilimumab and favorable clinical outcome (7, 8). In contrast, this correlation was neither observed in the current study, nor in recent investigations of large patient cohorts treated within early access programs (12, 49). Biomarkers predictive for severe autoimmunity are warranted as they might improve the individual risk/benefit assessment. An early increase of AEC was recently reported to correlate with the occurrence of irAEs (50), but no such property was observed for the biomarker signatures described here.

In conclusion, a baseline signature of low values of LDH, AMC, and MDSCs as well as high AEC, Tregs, and RLC in the peripheral blood is associated with favorable outcome of late-stage melanoma patients treated with ipilimumab. Investigation of the predictive impact of these biomarkers following ipilimumab and other treatments, e.g., PD-1 antibodies, is warranted.

Disclosure of Potential Conflicts of Interest

M.A. Postow reports receiving commercial research grants from Bristol-Myers Squibb, and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, and Caladrius. P.A. Ascierto reports receiving commercial research grants from Bristol-Myers Squibb, Roche-Genentech, and Ventana; and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, Merck Sharp & Dohme, Novartis, Roche-Genentech, and Ventana. M. Maio is a consultant/advisory board member for Astra Zeneca, Bristol-Myers Squibb, MSD, and Roche. B. Schilling reports receiving commercial research grants from and is a consultant/advisory board member for Bristol-Myers Squibb. D. Schadendorf reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, Merck, Novartis, and Roche. J.C. Hassel reports receiving commercial research grants from Bristol-Myers Squibb; other commercial research support and speakers bureau honoraria from Bristol-Myers Squibb, Novartis, MSD, and Roche; and is a consultant/advisory board member for Amgen, and MSD. T.K. Eigentler reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Bristol-Myers Squibb. J.D. Wolchok reports receiving commercial research grants from and is a consultant/advisory board member for Bristol-Myers Squibb; and is a coinventor on MDSC patent owned by MSKCC and licensed to Sermetrix. C. Blank reports receiving commercial research grants from Novartis, and is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, MSD, Novartis, Pfizer, and Roche. C. Garbe reports receiving commercial research grants from Bristol-Myers Squibb, Novartis, and Roche; and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, MSD, Novartis, and Roche. B. Weide reports receiving commercial research grants from, is a consultant/advisory board member for, and reports receiving travel reimbursement from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Martens, K. Wistuba-Hamprecht, M. Geukes Foppen, M.A. Postow, P. Wong, E. Romano, A. Khammari, B. Dreno, M. Capone, P.A. Ascierto, A.M. Di Giacomo, M. Maio, B. Schilling, A. Sucker, D. Schadendorf, J.C. Hassel, J.D. Wolchok, C. Blank, G. Pawelec, C. Garbe, B. Weide

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