Clinical aspects of immunotherapy and targeted therapy of advanced melanoma

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Eat.Sleep.Workout.Repeat

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Chapter 1

General introduction

Melanoma is an aggressive form of skin cancer developing from melanocytes, which can affect men and women of all ages. Melanoma typically occurs in the skin, but it may also occur on mucosal surfaces such as intestines, vulva, nasopharynx, sinuses and mouth. Rarely melanoma is found in the eye [1]. Of all types of skin cancer, melanoma causes the most skin cancer related deaths.

INCIDENCE AND SURVIVAL

Melanoma was diagnosed in nearly 6000 patients in the Netherlands in 2015 and as can be seen in Figure 1. the incidence of melanoma in the Netherlands is steadily increasing. In 2015 more than 800 patients died due to melanoma in the Netherlands. Survival from melanoma is mainly dependent on the stage of the disease at diagnosis (Figure 2). Stage of melanoma is based on the staging system as defined by the American Joint Committee on Cancer (AJCC) [2]. This staging system focusses on tumor thickness, mitotic rate, ulcerations, the presence of nodal metastases and distant metastases. Patients without distant metastases are classified as stage I-III, while patients with distant metastases are classified as stage IV. The focus of this thesis lies on stage IV melanoma.

IMMUNOTHERAPY FOR THE TREATMENT OF METASTATIC MELANOMA

In 2013 the editors of *Science* chose cancer immunotherapy as the breakthrough of the year, hereby showing the importance of the immune system to combat tumors. Already in 1863 Rudolf Virchow described the presence of lymphoid cells in cancerous tissue and hypothesized a connection between inflammation and cancer [3]. For decades it is now known that these lymphocytic infiltrates play a crucial role in patients' clinical outcome in not only melanoma, but in the majority of cancers [4-8]. Pioneering work in this field of research has been performed by Dr. Steven Rosenberg from the Surgery Branch (SB) of the National Institutes of Health (NIH), Bethesda, Maryland. Work from Rosenberg et al. showed that harvesting tumor-infiltrating lymphocytes (TIL), expanding them *ex-vivo* and reinfusing them into patients with metastatic cancers could induce clinical responses [9]. A process called adoptive cell transfer, or ACT. However, these positive effects are mainly limited to metastatic melanoma. The discovery of T-cell checkpoint molecules such as Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) and Programmed Death receptor 1 (PD-1) paved the way for a new form of immunotherapy [10, 11]. Several years after this discovery antibodies directed against these molecules were manufactured. Prior to 2010 the chemotherapeutic dacarbazine, and in some countries high-dose IL-2, were the only

registered treatments against metastatic melanoma. Median overall survival of patients treated with dacarbazine was only 6-9 months [12, 13]. In 2010 the fully human monoclonal antibody ipilimumab, targeting CTLA-4 on the activated T-cell showed, for the first time, a survival benefit in patients with metastatic melanoma [14, 15].

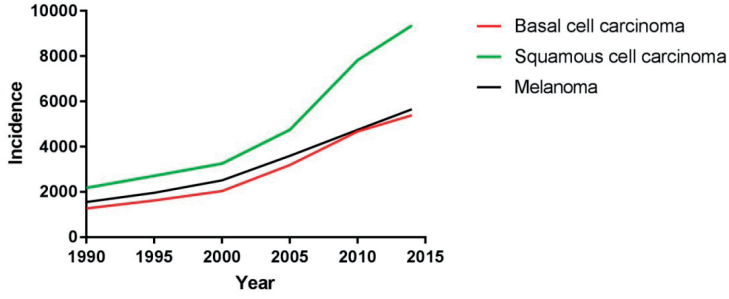

Figure 1. Incidence of skin cancer over the last 25 years (*Netherlands Cancer Registration*)

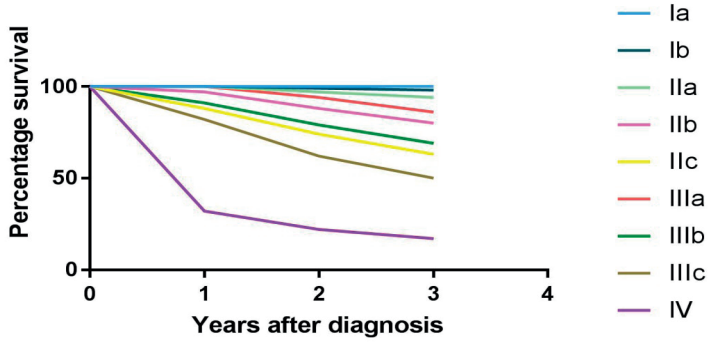

Figure 2. Mortality after diagnosis according to the AJCC staging system (*Netherlands Cancer Registration*)

Stage Ia: T1a, No, Mo; stage Ib: T1b/T2a, No, Mo; stage IIa: T2b/T3a, No, Mo; stage IIb: T3b/T4a, No, Mo; stage IIc: T4b, No, Mo; stage IIIa: T1-4a, N1a/N2a, Mo; stage IIIb: T1-4a/T1-4b, N1a/N2a/N1b/N2b/N2c, Mo; stage IIIc: T1-4b, N1b, N2b, N2c, N3, Mo; stage IV: all T, all N, M1.

This led to regulatory approval of ipilimumab for the treatment of metastatic melanoma. Roughly four years later pembrolizumab and nivolumab, both antibodies targeting PD-1, either as monotherapy or in combination with anti-CTLA-4 antibodies, showed even more impressive clinical results [16-18]. Median overall survival for patients with metastatic melanoma has since increased from 6-9 months with dacarbazine, to 10-20 months with ipilimumab to more than two years with anti-PD-1 antibodies as monotherapy or the com-

bination with an anti-CTLA-4 antibody. Despite these promising results, treating patients with these new antibodies has serious financial implications. The cost of treating a patient with four cycles of ipilimumab equals to about €90,000 (250 mg flat dose, for four cycles), while costs can run as high as €150,000 (240 mg flat dose, once every 2 weeks, for up to two years) for nivolumab and €260,000 (200 mg flat dose, once every 3 weeks for up to two years) for pembrolizumab [19]. Besides the financial aspects, some patients treated with these antibodies are at risk of serious, sometimes life-threatening adverse events (AEs), which are often immune-related (irAEs). For example, treatment related AEs of any grade in patients treated with ipilimumab can be seen in 89% of patients [14-16]. Although the majority of AEs was only grade I or II (the lower grades of AEs), 23% of patients had grade III or IV AEs (the higher grades of AEs). For patients treated with anti-PD-1 antibodies grade III/IV treatment related AEs are seen in up to 20% of patients, while patients treated with the combination of anti-PD1-antibodies and anti-CTLA4-antibodies grade III/IV treatment related AEs are seen in up to 59% [17, 18, 20-25]. Being able to select patients who will benefit the most from a certain treatment upfront remains one of the goals in cancer immunotherapy. Not only to reduce health-care costs, but mainly to steer patients into the right treatment, and thereby not treating patients with a certain immunotherapeutic agent that they are likely not to respond to. Until this date, several biomarkers have been discovered, but no biomarker (or combinations of biomarkers) has been incorporated into daily routine clinical practice. An example is serum lactate dehydrogenase (LDH). Three years ago, Kelderman et al. retrospectively showed that patients with a high LDH are less likely to respond to anti-CTLA-4 treatment. However, even at a serum LDH value of > 2 times the upper limit of normal a minority of patients still responded to this treatment [26]. Recently Blank et al. hypothesized a framework (the "cancer immunogram", Figure 3) consisting of seven parameters which could be crucial in anti-tumor response [27]. These seven parameters consist of: tumor foreignness, immune cell infiltration, absence of checkpoints, absence of soluble inhibitors, absence of inhibitory tumor metabolism, tumor sensitivity to immune effectors and general immune status. These parameters by themselves are all associated with response, or lack thereof, to immunotherapy. But what the cancer immunogram tries to show the treating physician is that it probably will not be just one single biomarker, but a combination of biomarkers which will make it possible to select patients upfront.

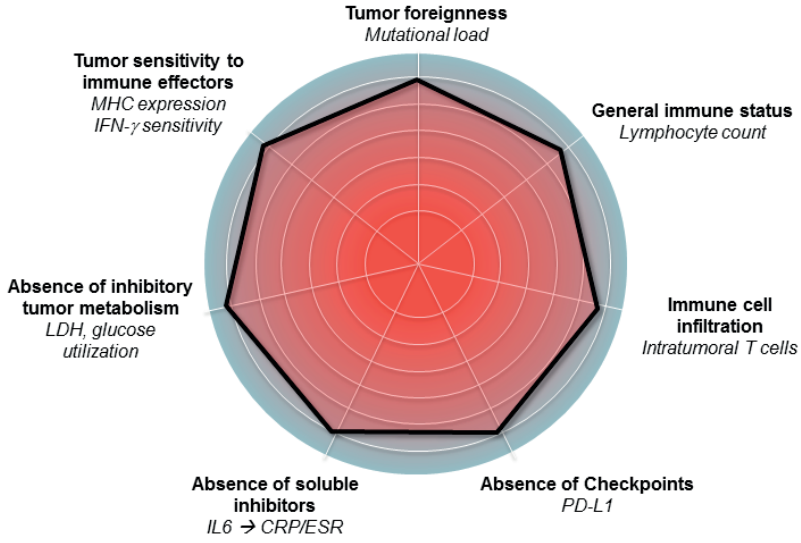

Figure 3. The "cancer immunogram"

BRAF INHIBITORS FOR THE TREATMENT OF METASTATIC MELANOMA

Approximately 40-60% of cutaneous melanoma harbor a mutation in the gene encoding *BRAF* [28, 29]. This mutation leads to constitutive activation of downstream signaling through the Mitogen-Activated Protein Kinase (MAPK) pathway. In approximately 80% of cases this mutation results in the substitution of valine by glutamic acid at codon 600 (V600E) [28, 29]. Other gene mutations, such as V600K and V600R are also known, but these mutations occur less frequently. Vemurafenib and dabrafenib are potent inhibitors of the mutated BRAF protein. Both have shown impressive objective response rates and improve progression free survival and overall survival when randomly compared to the chemotherapeutic dacarbazine in randomized phase III trials [30, 31]. Double targeting the MAPK pathway by combining BRAF inhibitors with MEK 1/2 inhibitors has clearly shown an improvement in not only efficacy, but also tolerability compared to BRAF inhibitor monotherapy [32-35].

CONCLUSION AND OUTLINE OF THE THESIS

Throughout melanoma history, significant progress has been made in treating patients with metastatic melanoma. This thesis will focus on different aspects of melanoma treatment with immunotherapy and targeted therapy.

In **chapter 2** we search for the perfect biomarker (or combination of biomarkers) to predict response to ipilimumab treatment. Here we look into different routine blood parameters, but also certain immune cell populations analyzed by flow cytometry. Identified parameters were first assessed in a discovery cohort and later validated in a validation cohort.

As previously mentioned a selection of patients treated with immunotherapeutics is at risk of developing adverse events, some of which can be life-threatening. One of those commonly seen adverse events is diarrhea. In **chapter 3** we retrospectively analyzed a cohort of 93 patients treated with immunotherapy for metastatic melanoma or non-small cell lung cancer. All patients underwent an endoscopy and/or were treated with high-dose corticosteroids for immune-related diarrhea. We describe the correlation between symptoms, endoscopic features, histological features and response to management.

In **chapters 4 and 5** we look into a select group of patients with metastatic melanoma. Namely, those with brain metastases and/or leptomeningeal metastases. The incidence of brain metastases ranges from 10% up to 73% based on clinical and post-mortem research [36-41]. Brain metastases from melanoma carry a poor prognosis with a median overall survival not exceeding five months [42]. In **chapter 4** we retrospectively analyzed a cohort of 146 patients with brain metastases from melanoma with a BRAF mutation. We describe the overall survival, progression free survival, clinical response and radiological response to BRAF inhibitors with or without the addition of a MEK inhibitor. In **chapter 5** we study patients with leptomeningeal metastases from metastatic melanoma. Literature has shown that patients with untreated leptomeningeal metastases from solid tumors have an even worse median overall survival of only 4 to 6 weeks [43]. In our retrospective analysis we identified a cohort of 39 patients with leptomeningeal metastases from melanoma and describe the effects of targeted therapy and immunotherapy on this disease.

BRAF inhibitors have proven to be an effective treatment against metastatic melanoma for patients harboring a BRAF mutation. However, a large proportion of patients treated with BRAF inhibitors will eventually relapse. In the clinical setting stopping the BRAF inhibitor after progression of disease oftentimes lead to an accelerated growth of the metastases, quickly followed by death of the patient.

In **chapter 6** we analyze two groups of 35 patients treated with the BRAF inhibitor vemurafenib. One group of patients continues with the BRAF inhibitor, despite documented progression of disease. The other group discontinues the BRAF inhibitor at documented progression. Here we describe the results of this analysis.

At the Netherlands Cancer Institute a phase III trial is in progress for patients with metastatic melanoma, comparing treatment with the adoptive transfer of TIL to ipilimumab. Patients receiving TIL are pre-treated with high-dose chemotherapy and receive high-dose bolus IL-2 after the infusion of the TIL. In **chapter 7** we review the past, present and future of treating patients with melanoma and other types of cancer with TIL.

Finally, in **chapter 8** the results obtained in this thesis are discussed and implications for further research are presented.

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Chapter 2

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 (MDSCs) and regulatory T cells (Tregs), 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. 43.5% of patients 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.

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, while 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.

Ipilimumab acts indirectly through immune cells by allowing T cell activation. $CD4^+$ T helper cells [15], $CD8^+$ cytotoxic T cells [16, 17], those targeting melanoma-associated- [18] or neo-antigens [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) [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) [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 (MDSCs) 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 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 (PBMCs). 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 1). 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 cut-off 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 cut-off points to divide the identification cohort for each factor into two or three groups. First, only central cut-off points were analyzed resulting in two balanced groups. A central cut-off point was considered for survival analysis if the resulting smaller group comprised at least 25% of all patients. Of all analyzed cut-off points, the lowest significant log-rank p-value was chosen as cut-off candidate 1. If no significant log-rank p-value was observed for any analyzed central cut-off, potential eccentric cut-offs (the resulting smaller group comprised at least 10% of patients) were analyzed. Of all analyzed eccentric cut-off points the lowest significant log-rank p-value was chosen as cut-off 1. For continuous variables with an established cut-off 1, the definition of a second cut-off point resulting in three groups according to this variable was attempted. A central second cut-off 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 cut-off points resulting in significant differences for each group-combination were further considered. Of those, the cut-off point resulting in the lowest significant log-rank p-value was chosen as cut-off 2. If no central second cut-off point could be established potential eccentric second cut-off 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 cut-off points resulting in significant differences for each group-combination were further considered. Of those, the cut-off point resulting in the lowest significant log-rank p-value was chosen as cut-off 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) [34]. A blinded or independent radiologic review was not conducted. The best overall response rate (BORR) 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 BORR was PR or CR and clinical benefit in case of SD, PR, or CR. Data on grade III, IV and V adverse events (AE) 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 adverse events (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 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, USA), and dead cells were excluded by ethidium monoazide labeling (EMA, Biotinum, USA). Staining was performed separately for the analysis of myeloid cells and T-cells/Tregs using antibody panels described in detail in Supplementary Table 1. Data were acquired with a BD LSR-II with FACS-Diva software V6.1.3 (BD, USA) and analyzed with FlowJo V9.3.2 (Tree Star, USA). Gating strategies are displayed in Supplementary Figure 1.

Statistical analysis

Overall survival 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 = 0.5 has no predictive value, a model with a c-index = 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 Chi square and Fisher´s exact tests. Throughout the analysis, p-values < 0.05 were considered statistically significant. Analyses were carried out using SPSS 22 (IBM, USA) and R 3.2.1 (R Foundation for Statistical Computing, Vienna Austria).

RESULTS

Patients and treatments

A total of 209 patients treated with ipilimumab at eight clinical sites was 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. 158 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%). 206 patients received at least one prior systemic treatment before ipilimumab. Of 198 with available data on the BORR 37 (18.7%) experienced a CR or PR. An additional 29 patients had SD, resulting in a clinical benefit rate of 33.3%. 160 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%). 77 (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 one patient. LDH was elevated in 184 (45.3%). 296 patients received all 4 doses, while in the remaining patients treatment was stopped after 1 – 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 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. 13 variables were associated with OS at one, and 7 at two, optimized cut-off points. In total, 27 variable/cut-off 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 cut-off points. In total, 10 biomarker/cut-off combinations derived from 8 biomarkers were confirmed and further considered. All variables, and survival analyses according to the cohorts and variable/cut-off combinations, are presented in Supplementary Table 2.

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)	

Table 1. Patient and treatment characteristics (*continued*)

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 (%)
Treatment background	CA-184-128 (3mg/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 (3mg /kg)	34 (32.4)	63 (60.6)	97 (46.4)	117 (28.8)
	Regular prescription (3mg/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.

Survival analysis using confirmed biomarkers

OS according to eight confirmed biomarkers (LDH and $Lin^{-}CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs at two cut-off points = 10 biomarker/cut-off 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-or > 2.3-fold, it was only 5 and 2 months, respectively ($P = 6.25x10^{-13}$; Figure 1A). A relative lymphocyte count (RLC) < 10.5% identified patients with a 1-year survival probability of only 5% ($P = 3.30x10^{-12}$; Figure 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 ($P = 6.73x10^{-11}$; Figure 1C). An absolute monocyte count (AMC) < 650/µL (Figure 1D) and a frequency of $CD14^{+}$ monocytes < 28% were also strongly associated with favorable outcome ($P = 1.35x10^{-08}$ and $6.58x10^{-07}$, respectively). Additionally, absolute (Figure 1E) and relative eosinophil counts (AEC and REC) were positively correlated with survival ($P = 5.06x10^{-05}$ and $2.14x10^{-04}$, respectively). Baseline frequencies of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs ≥ 1.5% were associated with good prognosis after initiation of ipilimumab ($P = 8.70x10^{-05}$; Figure 1F).

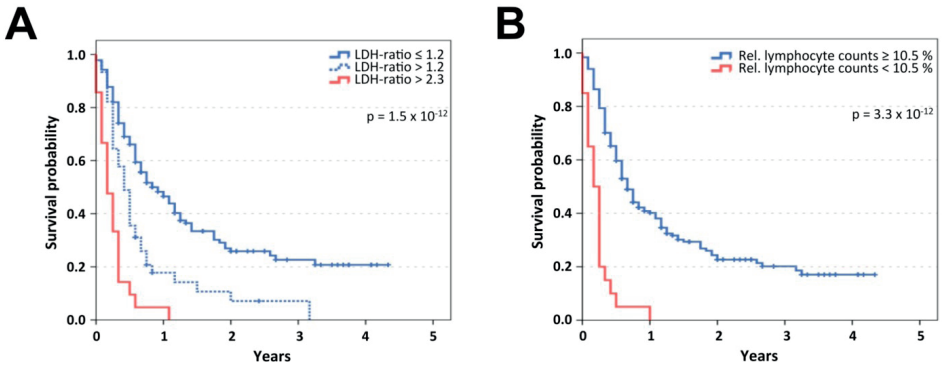

Figure 1. OS according to confirmed biomarkers. Kaplan-Meier analysis of OS in the identification and confirmation cohort ($n = 209$) according to LDH ratio (the measured LDH serum concentration divided by the upper limit of normal; A), RLC (B),

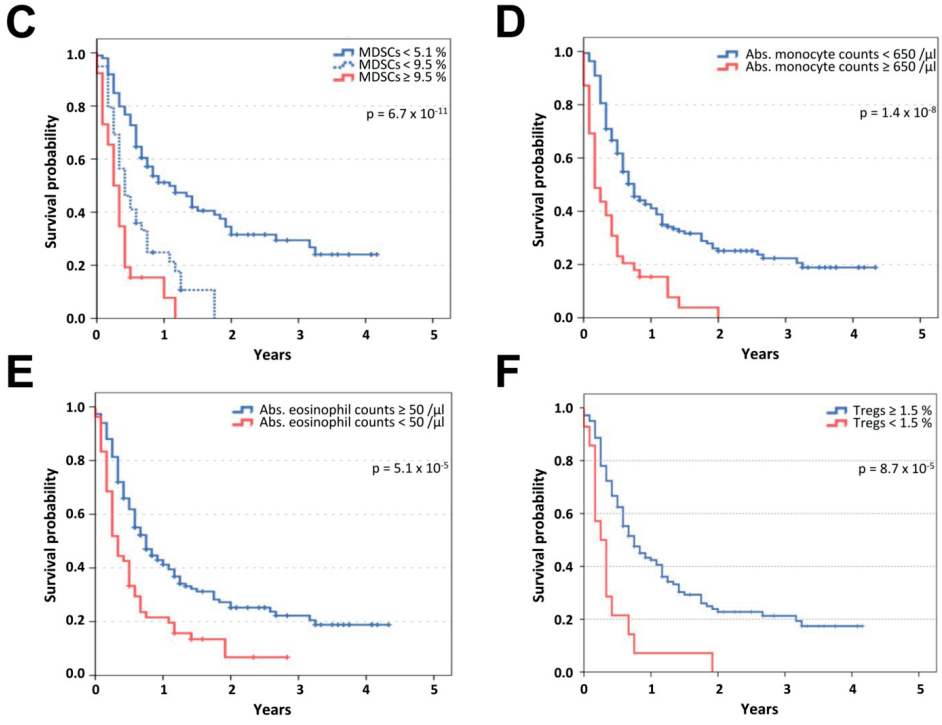

Figure 1. (continued) frequency of $Lin^{-}CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs (C), AMC (D), AEC (E), and frequency of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs (F). Censoring is indicated by vertical lines; *P* values were calculated by log-rank statistics.

Definition of a combination model

Cox regression analysis was performed to determine the relative impact of confirmed biomarkers. LDH (at both cut-off points), MDSCs, RLC, AMC, and AEC (each at one cut-off) remained in the model as significantly independent biomarkers. REC, Tregs, or $CD14^{+}$ monocyte frequencies did not 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 Figure 2A&B) and satisfactory calibration (Supplementary Figure 3A) was achieved when Tregs were likewise considered in addition to LDH (at both cut-off 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 cut-off points) including Tregs was selected for further analysis (combination model 1). Classification of patients in this model was

Table 2. OS according to confirmed biomarkers

Factor	Total n	Categories	n (%)	% Dead	Univariate survival analysis						
					Median survival (months)	1-Year survival rate (95% CI)		2-Year survival rate (95% CI)		3-Year survival 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-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.

based on a linear predictor score (risk score) accounting for the relative impact of each marker in the combination model (Figure 2A).

The 2-year survival rate for patients with favorable values for all 6 biomarkers (risk-score = 0) was 40.8% compared to 17.3% for those with risk scores ≤ 130. In contrast, none of the patients with risk scores > 130 survived longer than 15 months (Figure 2B). Moreover, the rate of clinical responses differed strongly between risk-score groups (Figure 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.

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* 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			
	≤ 1.2	1.0		≤ 1.2	1.0		Normal	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-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.

[a]Relative lymphocyte count, AMC, AEC, and REC.

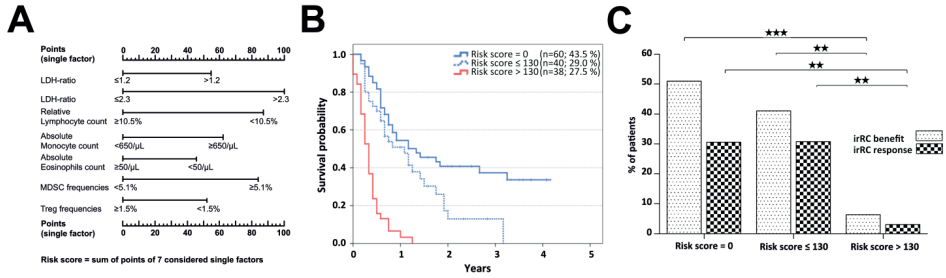

Figure 2. OS and tumor response according to combination model 1. A nomogram-based linear predictor measure was calculated for each patient considering the relative impact of single factors according to Cox regression analysis (A). In combination model 1, the LDH ratio (at two cutoff points), the absolute 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). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Definition of a combination model limited to routine markers

Next, we developed a less complex model which 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 vs. 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 Figure 2B) of all possible combination models considering the five routine markers (Supplementary Figure 2 C&D) and satisfactory calibration (Supplementary Figure 3B). The 2-year survival probability of patients with favorable profiles for all 4 markers was 43.1% compared to 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; Figure 3A). Similar to the first model, there was a strong correlation with the bOR (Figure 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.

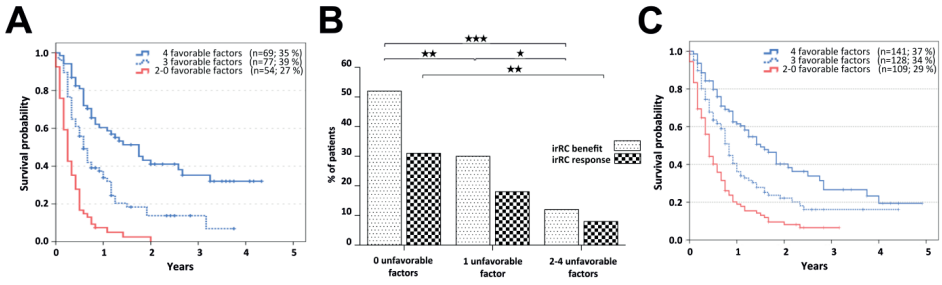

Figure 3. OS and tumor response according to combination model 2. In combination model 2, only routine biomarkers, available in daily practice, were considered. In addition to the absolute eosinophil and monocyte counts, the relative lymphocyte counts and LDH (categorized as elevated vs. normal) were 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). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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; Figure 3 C) 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 to 22.1% for those with one, and 9.5% for those with two or more unfavorable values.

Correlations with grade III/IV/V adverse events

Adverse events (AE) of grade III or higher were reported for 26 (12.6% of 207 evaluable patients) and immune-related adverse events (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 one patient, respectively. Severity of all AEs was classified as grade III and no grade IV or V toxicities were reported. The occurrence of AEs was neither cor-

related with OS since starting ipilimumab, nor with best clinical response, nor with the combination groups of baseline biomarkers (Supplementary Figure 4).

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 3).

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 Figure 5 B), and were overrepresented in the prognostically favorable biomarker groups (Supplementary Figure 5 A). 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 Figure 5 C&D).

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 impacting 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 et al. at the ASCO meeting 2013 [36] and an increase of eosinophils during ipilimumab was associated with OS in the study of Delyon [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 et al [10]. However, a different cut-off point used to categorize patients (300/µL *versus* 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 et al [31]. The inverse correlation of MDSC frequencies and OS following ipilimumab and the prognostic relevance for melanoma patients with distant metastasis in general [29] provides 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 [43-45]. MDSCs and Tregs have been shown to exert suppressive function on T cells, thereby possibly counteracting the beneficial effect of ipilimumab [28, 46].

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 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 three 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 three years and response rates of ~30%. In cases where several treatment options may be available for the individual patient, these findings may impact 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 vs. normal in the combined identification and confirmation cohorts.

Importantly, in this study we followed REMARK recommendations [47] and confirmed the association between ten variable/cut-off 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 which need to be considered. Other factors, for example the Eastern Cooperative Oncology Group (ECOG) performance status or prior treatments, for example with BRAF/MEK inhibitors, may impact 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 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 led 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.

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Conflicts of Interests:

T.K Eigentler reports receiving honoraria from BMS, travel/accommodations/expenses from Bristol-Myers Squibb and is a consultant/advisory board member for BMS. M. Maio reports receiving honoraria from BMS and Roche, reports receiving a commercial research grant from BMS, travel/accommodations/expenses from BMS, Roche and MSD and is a consultant/advisory board member for BMS and Roche. D. Schadendorf reports receiving honoraria from GSK, Roche, BMS, Amgen, Novartis, MSD, speakers bureau honoraria from GSK, Roche, BMS, Amgen, Novartis, MSD, reports receiving a commercial research grant from MSD, travel/accommodations/expenses from GSK, Roche, BMS, Amgen, Novartis, MSD and is a consultant/advisory board member for GSK, Roche, BMS, Amgen, Novartis, MSD. J.C. Hassel reports receiving honoraria from BMS, MSD, Roche, GSK, Novartis, Amgen. C. Blank reports receiving honoraria from BMS, MSD, GSK, Roche, Novartis, and a commercial research grant from Novartis. J. D. Wolchok reports receiving a commercial research grant from BMS, MSD. M. A. Postow reports receiving honoraria from BMS and a commercial research grant from BMS. J. Yuan reports receiving a commercial research grant from BMS. B. Schilling reports receiving a commercial research grant from BMS and travel/accommodations/expenses from BMS. C. Garbe reports receiving honoraria from BMS, MSD, Amgen, Novartis, Roche, GSK, reports receiving a commercial research grant from MSD, BMS, Roche, GSK. B. Weide reports receiving a commercial research grant

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SUPPLEMENTARY INFORMATION

Supplementary Table 1: Panels of antibodies used for flow cytometry

Panel	Specificity	Fluorochrome	Ab clone	Vendor
Myeloid-derived suppressor cells and monocytic cells	CD3 1	PerCP	SK7	BD
	CD3 2	BV605	OKT3	BioLegend
	CD4 1	PerCP	SK3	BD
	CD4 2	BV510	OKT4	BioLegend
	CD8 1	PerCP	SK1	BD
	CD11b 1 2	APC-Cy7	ICRF44	BD
	CD14 1 2	PE-Cy7	M5E2	BioLegend
	CD15 1 2	FITC	HI98	BD
	CD16 1 2	PB	3G8	BioLegend
	CD19 2	BV605	HIB19	BioLegend
	CD561	A700	B159	BD
	CD56 2	BV605	HCD56	BioLegend
	CD33 1	PE	HIM3-4	eBioscience
	CD124 1	APC	25463	R&D systems
	HLA-DR 1 2	PerCP-Cy5.5	G46-6	BD
T cells and regulatory T cells	CD3* 1	PO	UCHT1	Life Technologies
	CD3* 2	A700	UCHT1	BD
	CD4* 1	PerCP	SK3	BD
	CD4* 2	PE-Cy7	OKT4	BioLegend
	CD8* 1 2	APC-H7	SK1	BD
	CD25* 1 2	PE	M-A251	BD
	CD45RA* 1	BV421	HI100	BioLegend
	CD45RA* 2	PB	HI100	BioLegend
	CD103* 1	FITC	Ber-ACT8	BD
	CD103* 2	BV711	Ber-ACT8	BD
	CD127* 2	BV510	HIL-7R-M21	BD
	FoxP3 1 2	Alexa647	259DC7	BD
	Ki-67 2	FITC	20Raj1	eBioscience

Supplementary Table 1: Panels of antibodies used for flow cytometry. * Cells were fixed and permeabilized with FoxP3 buffer (BD). Only frequencies of CD14+ cells from MDSC panel 1 and frequencies of CD4+, CD8+ T cells, as well as their ratio, were included from Treg panel 1. [1] Panel 1 (N = 25), [2] Panel 2 (N = 184).

Supplementary Table 2: Spectrum of factors, cut-offs, and differences in overall survival according to biomarkers in the identification and the confirmation cohort

Group	Variable	Categories*	Univariate analysis of overall survival**			
			Identification cohort (N = 105)		Confirmation cohort (N = 104)	
			Log rank p value	Interpretation	Log rank p value	Interpretation
Clinical factors	Gender	Female vs. Male	9.27E-01	failed		
	Age	≤43 years vs. >43 years	3.99E-02	candidate	1.56E-01	failed
	Pattern of visceral tumor involvement	Soft-tissue and/or lung vs. other organs	1.81E-04	candidate	3.46E-01	failed
	Presence of brain metastases	yes vs. no	1.73E-01	failed		
Serum	LDH-ratio	≤1.2 vs. >1.2	2.88E-04	candidate	5.19E-07	confirmed
		≤2.3 vs. >2.3	9.71E-06	candidate	2.96E-06	confirmed
Blood count	Abs. leucocyte counts	<8150/µL vs. ≥8150/µL	6.30E-06	candidate	1.91E-01	failed
		<6250/µL vs. ≥6250/µL	1.92E-04	candidate	3.85E-01	failed
	Abs. lymphocyte counts	<1050/µL vs. ≥1050/µL	5.79E-02	failed		
	Rel. lymphocyte counts	<16.5% vs. ≥16.5%	6.03E-05	candidate	2.54E-01	failed
		<10.5% vs. ≥10.5%	2.20E-09	candidate	4.07E-05	confirmed
	Abs. monocyte counts	<450/µL vs. ≥450/µL	2.52E-06	candidate	4.89E-01	failed
		<650/µL vs. ≥650/µL	4.73E-06	candidate	9.59E-04	confirmed
	Rel. monocyte counts	<10.5% vs. ≥10.5%	4.82E-03	candidate	7.39E-01	failed
	Abs. eosinophil counts	<50/µL vs. ≥50/µL	2.75E-02	candidate	1.32E-05	confirmed
	Rel. eosinophil counts	<1.5% vs. ≥1.5%	2.10E-02	candidate	1.04E-03	confirmed

Supplementary Table 2: Spectrum of factors, cut-offs, and differences in overall survival according to biomarkers in the identification and the confirmation cohort (*continued*)

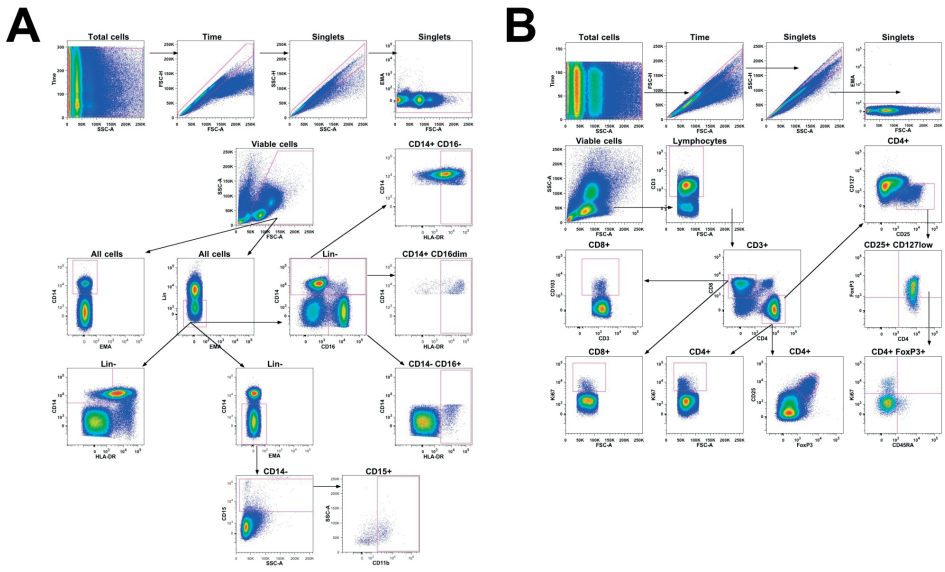
Group	Variable	Categories*	Univariate analysis of overall survival**			
			Identification cohort (N = 105)		Confirmation cohort (N = 104)	
			Log rank p value	Inter-pretation	Log rank p value	Inter-pretation
Immune cell subsets in the peripheral blood analyzed by flow cytometry	CD4+ T cells	<70% vs. ≥70%	2.53E-03	candidate	6.77E-01	failed
	CD8+ T cells	<23% vs. ≥23%	7.18E-03	candidate	6.19E-01	failed
	CD4/CD8 ratio	<3.0 vs. ≥3.0	3.61E-03	candidate	6.74E-01	failed
	CD8+CD103+ T cells	<0.8% vs. ≥0.8%	3.02E-01	failed		
	CD8+Ki67+ T cells	<3.6% vs. ≥3.6%	1.10E-02	candidate	1.84E-01	failed
	CD4+Ki67+ T cells	<0.7% vs. ≥0.7%	3.38E-02	candidate	6.87E-01	failed
	CD4+CD25+FoxP3+ Tregs	<1.5% vs. ≥1.5%	1.24E-03	candidate	3.78E-02	confirmed
	CD4+CD127lowCD25+FoxP3+ Tregs	<3.3% vs. ≥3.3%	1.44E-01	failed		
	CD4+CD127lowCD25+FoxP3+ CD45RA-Ki67+ proliferating Tregs	<0.3% vs. ≥0.3%	5.05E-03	candidate	2.57E-01	failed
		<0.2% vs. ≥0.2%	3.40E-03	candidate	7.14E-02	failed
	CD4+CD127lowCD25+FoxP3+ CD45RA+Ki67- non-proliferating Tregs	<0.2% vs. ≥0.2%	4.58E-01	failed		
	CD14+ monocytes	<20% vs. ≥20%	7.64E-07	candidate	5.56E-01	failed
		<28% vs. ≥28%	1.65E-07	candidate	2.34E-02	confirmed
	Lin-CD14+HLA-DR-/low MDSCs	<5.1% vs. ≥5.1%	1.03E-08	candidate	2.20E-03	confirmed
		<9.5% vs. ≥9.5%	3.41E-08	candidate	2.87E-03	confirmed
	Lin-CD14+CD16-HLA-DR+ classical monocytes	<10.4% vs. ≥10.4%	2.22E-02	candidate	7.52E-01	failed
	Lin-CD14-CD16+HLA-DR+ non-classical monocytes	<0.9% vs. ≥0.9%	1.78E-01	failed		
	Lin-CD14+CD16+HLA-DR+ monocytes	<0.7% vs. ≥0.7%	4.09E-05	candidate	2.66E-01	failed
	Lin-CD14-CD15+CD11b+ MDSCs	<0.2% vs. ≥0.2%	4.88E-01	failed		

Absolute (Abs.), Relative (Rel.), Lactate dehydrogenase (LDH), regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs). * Green characters indicate the category associated with better survival in the identification cohort. ** Green cells indicate significant differences in overall survival ($P < 0.05$). Red cells indicate non-significant findings.

Supplementary Table 3: Multivariate Models including only patients receiving 3mg/kg ipilimumab

	Multivariate analysis of significantly independent factors (N = 135)			Multivariate analysis including Tregs (combination model 1) (N = 135)			Combination model 2 considering LDH (elevated vs. normal) and blood count parameters* only (N = 193)		
Factor	**Category**	**HR**	**p-value**	**Category**	**HR**	**p-value**	**Category**	**HR**	**p-value**
LDH-ratio	> 2.3	5.3	0.0131	> 2.3	5.4	0.0085	Elevated	1.9	0.0003
	> 1.2	1.9	0.0214	> 1.2	1.8	0.0268			
	≤ 1.2	1.0		≤ 1.2	1.0		Normal	1.0	
Relative lymphocyte counts	< 10.5%	2.5	0.0077	< 10.5%	2.7	0.0047	< 10.5%	4.4	<0.0001
	≥ 10.5%	1.0		≥ 10.5%	1.0		≥ 10.5%	1.0	
Absolute monocyte counts	≥ 650/µL	1.9	0.0337	≥ 650/µL	1.8	0.0424	≥ 650/µL	2.1	0.004
	< 650/µL	1.0		< 650/µL	1.0		< 650/µL	1.0	
Absolute eosinophil counts	< 50/µL	1.6	0.0384	< 50/µL	1.6	0.0491	< 50/µL	1.7	0.0046
	≥ 50/µL	1.0		≥ 50/µL	1.0		≥ 50/µL	1.0	
Relative eosinophil counts	< 1.5%	Not independent		< 1.5%	Not considered		< 1.5%	Not independent	
	≥ 1.5%			≥ 1.5%			≥ 1.5%		
Lin-CD14+HLA-DR-/low MDSCs	≥ 9.5%	Not independent		≥ 9.5%	Not considered		Not considered		
	≥ 5.1%	2.5	0.0001	≥ 5.1%	2.4	0.0002			
	< 5.1%	1.0		< 5.1%	1.0				
CD4+CD25+FoxP3+ Tregs	< 1.5%	Not independent		< 1.5%	1.8	0.1233			
	≥ 1.5%			≥ 1.5%	1.0				
CD14+ monocytes	< 28%	Not independent		< 28%	Not considered				
	≥ 28%			≥ 28%					

Lactate dehydrogenase (LDH), regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), hazard ratio (HR). * Relative lymphocyte count, absolute monocyte count, absolute and relative eosinophil count.

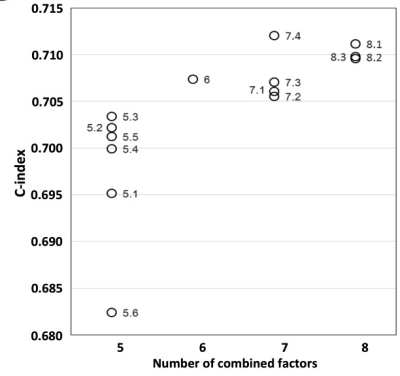


Supplementary Figure 1: Detailed gating strategy for quantification of subsets of monocytes and myeloid-derived suppressor cells (MDSCs), T cells and regulatory T cells (Tregs). Total cells were selected by gating on Time vs. SSC-A. Duplicates were removed via progressive gating on FSC-H vs. FSC-A and SSC-H vs. SSC-A. Dead cells were excluded by considering only EMA-negative cells. (A) A lineage cocktail (CD3, CD19, CD56) was used to avoid cross-contamination. Previously described MDSC populations were identified as $Lin^{-}CD14^{+}HLA\text{-}DR^{low}$ and $Lin^{-}CD14^{-}CD15^{+}CD11b^{+}$ within the all-cell gate. Overall monocytes were defined as $CD14^{+}$, while subsets were separated into classical monocytes ($Lin^{-}CD14^{+}CD16^{-}HLA\text{-}DR^{+}$), non-classical monocytes ($Lin^{-}CD14^{-}CD16^{+}HLA\text{-}DR^{+}$) and Lin^{-} $CD14^{-}CD16^{dim}HLA\text{-}DR^{+}$ monocytes within the all-cell gate. (B) A morphological gate was used to identify the population of lymphocytes. Next, $CD3^{+}$ cells were selected and further separated into $CD4^{+}$ and $CD8^{+}$ cells. Ki67 expression was investigated on $CD4^{+}$ and $CD8^{+}$ cells. $CD8^{+}$ T cells with suppressive potential were defined as $CD103^{+}$. Previously described phenotypes of Tregs were defined as $CD4^{+}CD25^{+}FoxP3^{+}$ and $CD4^{+}CD127^{low}CD25^{+}FoxP3^{+}$. These were further subdivided into proliferating ($Ki67^{+}CD45RA^{-}$) and non-proliferating Tregs ($Ki67^{-}CD45RA^{+}$).

A

Model	LDH-ratio >2.3	LDH-ratio >1.2	Relative lymphocyte counts <10.5%	Absolute monocyte counts ≥650/µL	Absolute eosinophil counts <50/µL	Lin- CD14+HLA-DR-/low MDSCs ≥5.1%	Relative eosinophil counts <1.5%	CD4+CD25+ FoxP3+ Tregs <1.5%	Lin- CD14+HLA-DR-/low MDSCs ≥9.9%	CD14+ Monocytes < 28%
5.1	x		x	x	x	x				
5.2		x	x	x	x	x				
5.3	x	x		x	x	x				
5.4	x	x	x		x	x				
5.5	x	x	x	x		x				
5.6	x	x	x	x	x					
6	x	x	x	x	x	x				
7.1	x	x	x	x	x	x				x
7.2	x	x	x	x	x	x	x			
7.3	x	x	x	x	x	x			x	
7.4	x	x	x	x	x	x		x		
8.1	x	x	x	x	x	x		x	x	
8.2	x	x	x	x	x	x	x	x		
8.3	x	x	x	x	x	x		x		x

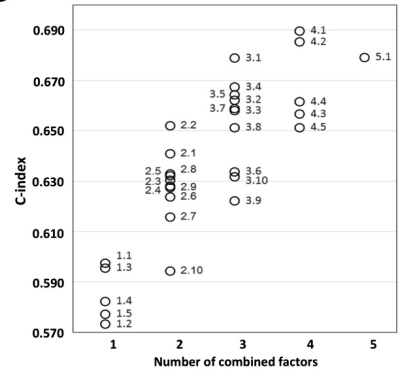
B



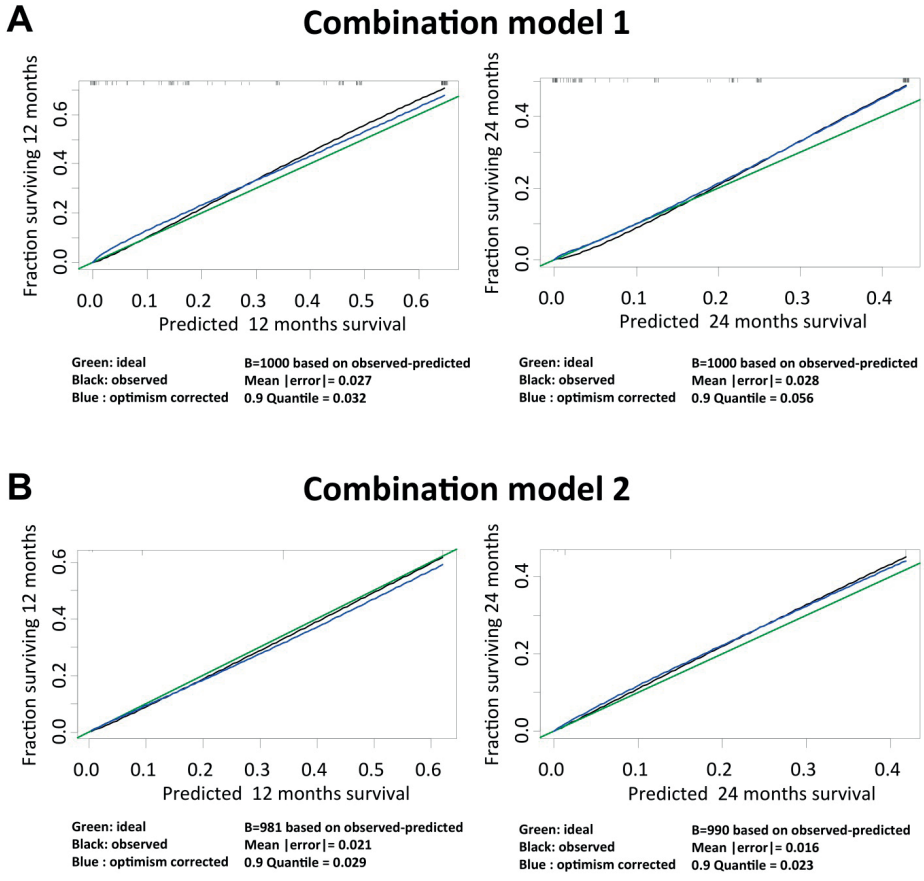
C

Model	LDH-ratio elevated	Relative lymphocyte counts <10.5%	Absolute monocyte counts ≥650/µL	Absolute eosinophil counts <50/µL	Relative eosinophil counts <1.5%
1.1	x				
1.2		x			
1.3			x		
1.4				x	
1.5					x
2.1	x	x			
2.2	x		x		
2.3	x			x	
2.4	x				x
2.5		x	x		
2.6		x		x	
2.7		x			x
2.8			x	x	
2.9			x		x
2.10				x	x
3.1	x	x	x		
3.2	x	x		x	
3.3	x	x			x
3.4	x		x	x	
3.5	x		x		x
3.6	x			x	x
3.7		x	x	x	
3.8		x	x		x
3.9		x		x	x
3.10			x	x	x
4.1	x	x	x	x	
4.2	x	x	x		x
4.3	x	x		x	x
4.4	x		x	x	x
4.5		x	x	x	x
5.1	x	x	x	x	x

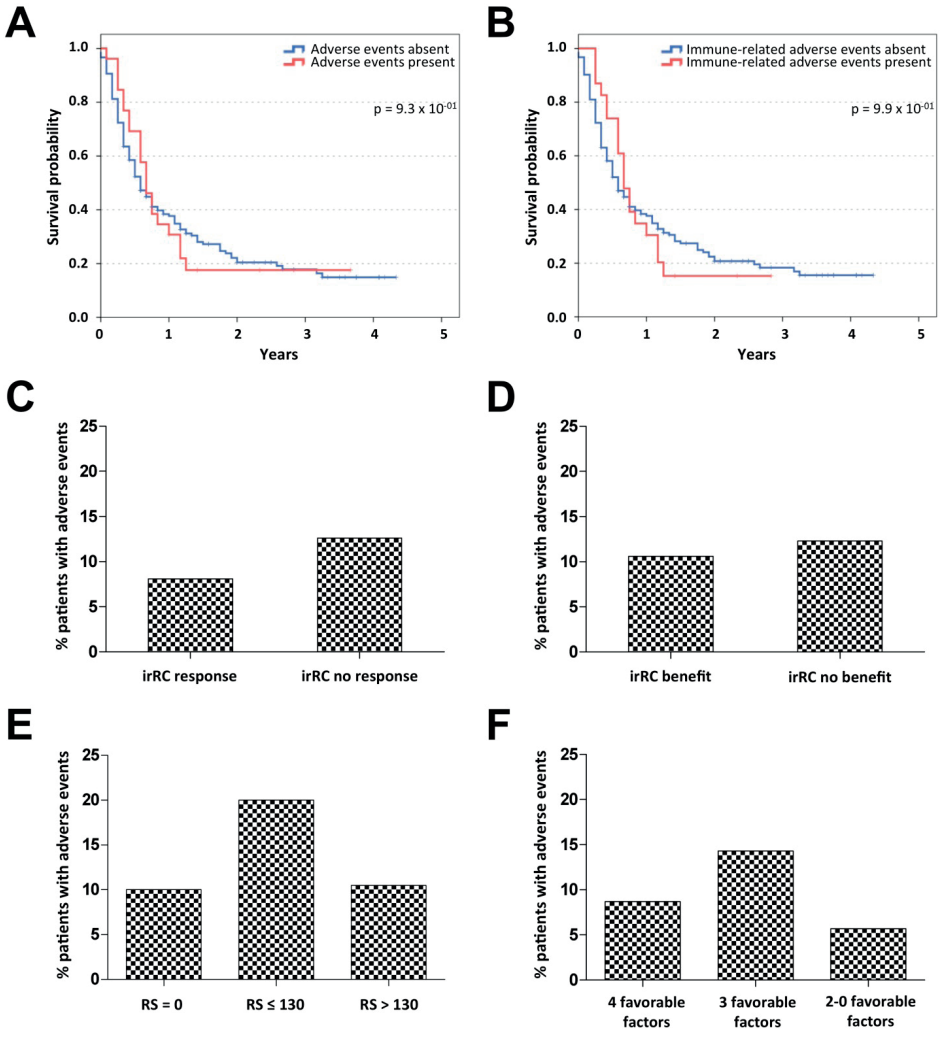
D



Supplementary Figure 2: Discriminatory ability of combination models. The concordance index (c-index, y-axis) was calculated for the combination of factors with independent impact according to Cox regression analysis (model 6.1) and 13 alternative combination models considering 5, 7, or 8 factors (A). The numbers refer to the rows in A. The c-indices are presented according to the number of combined factors (B). The combination model with highest discriminatory ability (7.4), which considered regulatory T cells in addition to the 6 factors with independent impact according to Cox regression analysis was chosen as combination model 1. No further increase of the c-index compared to combination model 1 was observed if one of the 3 remaining factors was additionally considered (models 8.1, 8.2, 8.3). C-indices were calculated for different combination models accounting for the number of unfavorable values of all factors considered in the given model (C). All possible models derived from combinations of the five routine factors were considered. The c-indices are presented according to the number of considered factors (D). The model with highest discriminatory ability (4.1) was selected.



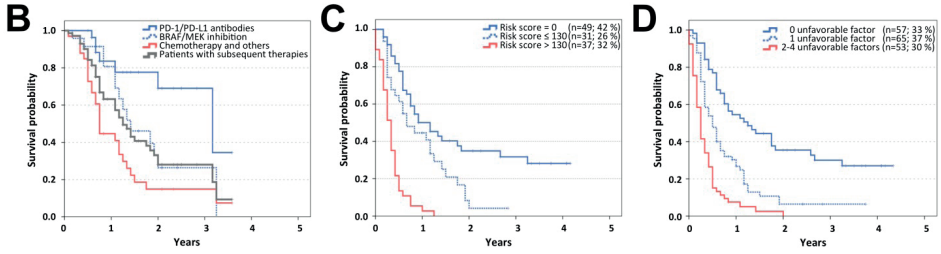
Supplementary Figure 3: Calibration of combination models. Calibration was calculated after 12 and 24 months using the calibrate function in the rms package of R for combination model 1 (A) and combination model 2 (B). Bootstrapping (1000 repeats) was performed to obtain bias-corrected estimates of predicted vs. observed values. Non-convergence reduced the number of included bootstrapping steps for combination model 2 to 981 or 990 after 12 or 24 months, respectively. "Predicted" survival probabilities at 12 or 24 months are those predicted by the Cox model, and "observed" refers to the corresponding Kaplan-Meier survival estimate at the given time-point. Mean absolute error in predictions, the mean squared error, and the 0.9 quantile of the absolute error is reported. "Error" refers to the difference between the predicted values and the corresponding bias-corrected calibrated values. Mean error was < 3% for both combination models and both time-points. The calibration according to Kolmogorov Smirnov was excellent for combination model 1 and satisfactory for model 2 (P = 0.657 and P = 0.021, respectively).



Supplementary Figure 4: Correlations between adverse events and overall survival, clinical response, or biomarker categories. Overall survival was not different between patients stratified according to the occurrence of adverse events (AEs) in general (A) or immune-related AEs (irAEs). (B). Kaplan-Meier analysis is presented and censoring is indicated by vertical lines; p-values were calculated by log rank statistics (A&B). No correlations were observed between the occurrence of irAEs during ipilimumab treatment and the best tumor response (C, D) nor with the proposed combination groups of baseline biomarkers according to the combination model 1 (E) or combination model 2 (F). The best overall tumor response according to immune-related response criteria (irRC) was analyzed either as the rate of patients with an irRC response (sum of those with complete or partial responses) or irRC benefit (sum of those with complete responses, partial responses and stable disease). Differences were not statistically significant.

A

Subsequent treatment with PD-1/PD-L1 antibodies	Combination model 1 (n=47)			Combination model 2 (n=67)		
	risk score = 0 (n; %)	risk score ≤ 130 (n; %)	risk score > 130 (n; %)	0 unfavorable factor (n; %)	1 unfavorable factor (n; %)	2-4 unfavorable factors (n; %)
Yes	11; 52.4%	9; 42.9%	1; 4.8%	12; 48.0%	12; 48.0%	1; 4.0%
No	11; 42.3%	10; 38.5%	5; 19.2%	14; 33.3%	16; 38.1%	12; 28.6%



Supplementary Figure 5: Overall survival and distribution after first dose of ipilimumab according to subsequent treatments. Of 209 patients, 71 received at least one additional systemic line of treatment after ipilimumab. 137 individuals did not receive further therapy and data were not available for one patient. 47 (combination model 1) or 67 (combination model 2) of 71 patients had complete data for classification according to biomarker combination models. The representation of PD-1/PD-L1-treated patients in the biomarker groups was shifted towards favorable biomarker combination groups for both combination models compared to those without subsequent PD-1/PD-L1 treatment. Therefore, a confounding effect of subsequent treatment with PD-1/PD-L1 antibodies on the biomarker results of this study cannot be ruled out (A). To investigate the potential confounding impact on OS and biomarker findings, subsequent treatments were categorized into three different groups: BRAF/MEK inhibitors (N = 24), PD-1/PD-L1 antibodies (N = 28), and chemotherapy/other treatments (N = 33) and analyzed by the Kaplan-Meier method (B). Patients treated with PD-1/PD-L1 antibodies had a significant better survival compared to all 71 patients (P = 0.006), while no significant difference was observed for the other two groups. Kaplan Meier analysis of overall survival of patients classified according to combination model 1 (C) or combination model 2 (D) is presented after exclusion of individuals who received subsequent treatment with anti-PD-1 or PD-L1 antibodies, as a confounding effect could not be ruled out. However, the prognostic impact of the proposed biomarker combinations at baseline of ipilimumab treatment remained strong (P < 0.018 for all pairwise comparisons of categories of the respective model). Censoring is indicated by vertical lines. Programmed cell death protein-1 (PD-1), programmed cell death protein ligand-1 (PD-L1), Risk score (RS).

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Chapter 3

Immune-checkpoint inhibition-related colitis: Symptoms, endoscopic features, histology and response to management

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Background

Immune-checkpoint-inhibitors are successfully introduced as anti-cancer treatment. However, they may induce severe immune-related adverse events (irAEs). One of the most frequent irAEs is diarrhea. The main objective of this study was to analyze symptoms (i.e. grade of diarrhea), endoscopic and histological features and response to management in immune-checkpoint inhibition-related colitis (IRC).

Patients and methods

We retrospectively analyzed patients who developed diarrhea upon checkpoint inhibition and therefore underwent an endoscopy and/or were treated with corticosteroids. Patients were treated between August 2010 and March 2016 for metastatic melanoma or non-small cell lung cancer. Severity of IRC was scored using the endoscopic Mayo score and the van der Heide score.

Results

Out of a cohort of 781 patients ninety-two patients were identified who developed diarrhea and therefore underwent an endoscopy and/or were treated with corticosteroids. Patients were treated with monotherapy anti-CTLA-4, anti-PD-1, or a combination of both. All patients had symptoms of diarrhea (16% grade 1, 39% grade 2 and 44% grade 3). A complete colonoscopy was performed in 62 (67%) patients, of whom 42 (68%) had a pancolitis (≥ 3 affected segments). Ulcers were seen in 32% of endoscopies. There was no significant correlation between the grade of diarrhea at presentation and endoscopic severity scores, the presence of ulcers or histological features. In 54 episodes of diarrhea (56%) patients received one or more cycles infliximab for steroid-refractory colitis. Patients with higher endoscopic severity scores, ulcers and/or a pancolitis needed infliximab more often.

Conclusions

The correlation between grade of diarrhea and endoscopic or histological features for severity of colitis is poor. Patients with higher endoscopic severity scores, ulcers, or a pancolitis, needed the addition of infliximab more often. Therefore, endoscopy may have value in the evaluation of the severity of immune-checkpoint inhibitor-related colitis and may help in decision making for optimal management.

Significance of this study

What is already known about this subject?

- Immunotherapy can induces adverse events, which are predominantly immune-related.
- One of the most common and severe immune-related adverse events is diarrhea.
- Diarrhea is seen in 35% of patients treated with anti-CTLA-4, 20% in patients treated with anti-PD-1 and even 44% in patients treated with the combination therapy.

What does this study add?

- Patients in which ulcers were seen during endoscopy required significantly more often the addition of infliximab for steroid-refractory colitis compared to patients in which no ulcers were seen.
- Patients with a high Van der Heide score, a high Mayo score or a pancolitis required significantly more often the addition of infliximab for steroid-refractory colitis compared to patients with a low Van der Heide score, low Mayo score or no pancolitis.
- There was no significant correlation between the grade of diarrhea at presentation and endoscopic Mayo score, van der Heide score, or presence of ulcers.
- There was no correlation between the presence of abdominal pain and any endoscopic feature.
- The most common histopathological feature was an increase in lamina propria cellularity, primarily consisting of mononuclear cells. The second most common histopathological feature was neutrophilic infiltration, either intraepithelial or as crypt abscesses.

How might this impact on clinical practice?

- Algorithms to guide management of immune-related diarrhea should not be based on the grade of diarrhea.
- Endoscopic features, such as the presence of ulcers or a pancolitis, can help clinicians to intensify immune suppression more rapidly.
- Histopathology does not seem to have an added value to guide therapy beyond what is found endoscopically. Mucosal biopsies appear to mainly serve to confirm diagnosis.

INTRODUCTION

The introduction of immune-checkpoint inhibitors has changed treatment options and improved survival of patients with advanced cancer. Ipilimumab, a monoclonal antibody blocking cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T cells, showed an overall survival benefit in patients with advanced melanoma [1]. Nivolumab and pembrolizumab, both antibodies blocking programmed death-receptor 1 (PD-1), improved survival compared to chemotherapy and ipilimumab [2, 3]. The combination of ipilimumab with an anti-PD-1 antibody improves overall response rate and progression free survival even further compared to single agent therapy [4]. Checkpoint inhibitors also show activity in several other types of cancer, such as metastatic non-small cell lung cancer (NSCLC) and bladder cancer [5-7]. Although efficacy and durability of response with checkpoint inhibitors has been well established, one of the major concerns is the high rate of adverse events that are predominantly immune-related.

Diarrhea

One of the most common and severe immune-related adverse events (irAEs) is diarrhea, with an incidence of 35% for anti-CTLA-4, 20% for anti-PD-1 and even 44% for the combination therapy [4, 8]. The median time to onset of diarrhea is 7 – 8 weeks after start for ipilimumab (or combinations with ipilimumab), compared to 3 – 6 months for anti-PD-1 [9-12]. The Common Terminology Criteria for Adverse Events (CTCAE version 4.03) are often used to define grades of diarrhea in patients treated in clinical trials. Grade 1 diarrhea is defined as an increase of < 4 stools over baseline, grade 2 as between 4 – 6 stools over baseline, grade 3 as ≥ 7, grade 4 as life-threatening consequences and grade 5 as death.

Treatment-algorithms

Current treatment-algorithms for immune-checkpoint inhibition-related colitis (IRC) are based on symptoms of diarrhea graded according to CTCAE [13-16]. For patients with grade 2 diarrhea delay of immunotherapy and start of symptomatic treatment with loperamide is considered. If symptoms persist for > 3 days, oral corticosteroids in a dose of 0.5 – 1.0 mg/kg are recommended. For patients with grade 3 or 4 diarrhea, discontinuation of immunotherapy (IT) and treatment with 1.0–2.0 mg/kg prednisone is advised. Steroid-refractory colitis is defined as the persistence of symptoms within 3 days of high-dose corticosteroids. These patients could be treated with the addition of 5 mg/kg infliximab. The implementation of these treatment-algorithms has resulted in a decrease of serious complications such as perforation and colectomy [17]. According to these algorithms a lower endoscopy is advised for patients with grade 3 or 4 symptoms of diarrhea, but no recommendations are provided on differential treatment based on endoscopic findings. The aim of this study was to try to correlate symptoms, endoscopic features, histology and

response to management in patients that developed diarrhea upon immune-checkpoint inhibition.

METHODS

Patients

Patients who developed diarrhea upon immunotherapy and therefore underwent an endoscopy and/or were treated with corticosteroids, were retrospectively identified. All patients were treated for melanoma or NSCLC, between August 2010 and March 2016. Patients were treated with monotherapy anti-CTLA-4, anti-PD-1, a combination of both, or the combination of anti-CTLA-4 and radiofrequency ablation (RFA). Diarrhea was scored according to CTCAE version 4.03. All patient characteristics were derived from the electronic patient records. Routinely, stools were tested for microorganisms, including SSYC, *Clostridium difficile* and viral pathogens. Severity of IRC on endoscopy was scored retrospectively using two different scoring systems (Supplementary Table 1). Endoscopic characteristics of IRC are very diverse and there are no available validated scoring systems. Often, a diffuse component of inflammation was present and therefore we used the Mayo score, which is validated for scoring diffuse inflammation seen in ulcerative colitis (UC) [18]. However, this score is not ideal in patients with ulcers among a normal or slightly friable mucosa. When ulcers were present in a further normal mucosa a Mayo score of 0 with a positive ulcer score was given in our study. We also used the van der Heide score, as it is more descriptive and therefore potentially more useful for the diverse characteristics seen in IRC. This score has been used previously for this purpose [19, 20]. However, the van der Heide score does not take into account the extensiveness of inflammation. Therefore, numbers of affected segments of the colon (recto-sigmoid, descending, transverse and ascending) were scored separately. Involvement of ≥ 3 segments was defined as pancolitis. Scores were gathered through saved images and endoscopy reports and revised by one gastroenterologist (JvD), blinded for the grade of diarrhea. As the scores may be influenced by subjectivity, the most objective endoscopic feature, namely the presence of ulcers, was analyzed as a separate variable. An ulcer was defined as a mucosal break of ≥ 0.5 centimeter. All hematoxylin-eosin (HE) stained slides of biopsies taken during endoscopies were reassessed by one gastrointestinal pathologist (PS).

Treatments

Patients treated with ipilimumab, nivolumab or pembrolizumab as monotherapy received standard or flat doses. Patients who received the combination of ipilimumab and RFA (radiofrequency ablation) underwent RFA of one liver metastasis, directly followed by four cycles of ipilimumab (depending on the cohort, either 3 mg/kg or 10 mg/kg q3 weeks). Pa-

tients received either the standard combination of ipilimumab (3 mg/kg) and nivolumab (1 mg/kg) or a sequential but overlapping scheme of 2 cycles ipilimumab 3mg/kg on day 1 and 22 followed directly by nivolumab (3mg/kg) or pembrolizumab (2mg/kg) from day 23 and onwards q2 weeks or q3 weeks respectively.

Statistical analysis

For continuous variables data are presented as median with interquartile range (IQR) and categorical variables as a number (%). Correlations between clinical symptoms and the endoscopic features were assessed using Spearman rank correlation coefficient. Associations between clinical symptoms, endoscopic features, histology and outcome of management were analyzed by Chi-square tests. A *P* value of < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 22 (IBM corp., Armonk, NY, USA).

RESULTS

Patient characteristics

Out of a cohort of 781 patients ninety-two patients were identified who developed diarrhea and therefore underwent an endoscopy and/or were treated with corticosteroids. All patient characteristics have been summarized in Table 1. Four patients had two different episodes of diarrhea (median days between episodes 318 days; range 190–632). Mean age was 58 years (range 30–88) and 54% of patients were female. Eighty patients were treated for metastatic melanoma (87%) and 12 patients (13%) for metastatic NSCLC. Fifty-six percent (54/96) of episodes were due to anti-CTLA-4 (of which 10/54 received the combination with RFA), 22% due to anti-PD-1 and 22% due to the combination of anti-CTLA-4 and anti-PD-1. In sixteen percent of episodes patients had grade 1 diarrhea, 39% grade 2 diarrhea and 44% grade 3 diarrhea. In 48 episodes (50%) patients also experienced abdominal pain and in 29 episodes (30%) patients had bloody stools. Infectious causes for diarrhea were ruled out in 68 episodes (71%). Three patients had a positive stool culture for which they were treated with antibiotics. However, as symptoms did not resolve, an IRC component was present as well. The median time between the first cycle of immunotherapy and onset of diarrhea was 38 days (IQR 23 –62). For patients treated with ipilimumab the median time to onset of diarrhea was 33 days, for anti-PD-1 84 days and for the combination 27 days. Three patients developed a perforation of the colon, for which they underwent surgery (Supplementary Table 2). No patients died due to colitis.

Table 1. Patient characteristics

	No. (%)
Age median (range)	**58 (30 – 88)**
Gender	
Male	**42 (46)**
Female	**50 (54)**
Type of cancer	
Melanoma	**80 (87)**
NSCLC	**12 (13)**
Immunotherapy among 96 episodes	
Ipilimumab (3 mg/kg)	**44 (46)**
Ipilimumab (10 mg/kg)	**10 (10)**
Nivolumab	**11 (12)**
Pembrolizumab	**10 (10)**
Sequential ipilimumab + pembrolizumab	**7 (7)**
Sequential ipilimumab + nivolumab	**2 (2)**
Combined ipilimumab + nivolumab	**12 (13)**
Diarrhea at presentation among 96 episodes	
Grade 1	**15 (16)**
Grade 2	**37 (39)**
Grade 3	**43 (44)**
Grade 4-5	**0 (0)**
Unknown	**1 (1)**
Prednisone at start of diarrhea	
None	**4 (4)**
< 1 mg/kg	**32 (33)**
1 mg/kg	**57 (60)**
> 1 mg/kg	**3 (3)**
Budesonide	
No	**84 (87)**
Yes	**12 (13)**
Infliximab	
No	**42 (44)**
Yes	**54 (56)**
Mycophenolic acid	
No	**93 (97)**
Yes	**3 (3)**
Tacrolimus	
No	**94 (98)**
Yes	**2 (2)**

NSCLC: non-small cell lung cancer

Endoscopic results

In all but 3 episodes an endoscopy was performed. Endoscopy images were not available in one episode. These 4 patients were excluded from endoscopic and histopathologic analysis. The median time between start of diarrhea and endoscopy was 8 days (IQR 5 – 14), the median endoscopic Mayo score was 1 (range 0–3) and the median van der Heide score was 6 (range 0–12). Ulcers were seen during 29 endoscopies (32%). In the majority of endoscopies (79%) a continuous pattern of inflammation was seen. A complete colonoscopy was per-

formed in 62 (67%) patients, of whom 42 (68%) had a pancolitis (≥ 3 affected segments). No serious side-effects of colonoscopy were seen in our patients. All endoscopic features are summarized in Table 2. There was no significant correlation between grade of diarrhea

Table 2. van der Heide and endoscopic Mayo scores from 92 endoscopies

Endoscopic feature according to the van der Heide classification	No. (%)
Color	
Normal	**12 (13)**
Red	**58 (63)**
Deeply red	**22 (24)**
Vascular patter	
Normal	**18 (20)**
Partially absent	**45 (49)**
Completely absent	**29 (31)**
Friability	
Normal	**17 (19)**
Slightly friable	**48 (52)**
Severely friable	**27 (29)**
Granularity	
Absent	**23 (25)**
Fine granularity	**62 (67)**
Coarse granularity	**7 (8)**
Rectal valves	
Sharp	**46 (50)**
Swollen	**46 (50)**
Absent	**0 (0)**
Ulcers	
Absent	**63 (69)**
Few	**18 (19)**
Multiple	**11 (12)**
Spontaneous bleeding	
Absent	**87 (95)**
Discrete	**4 (4)**
Severe	**1 (1)**
Mucopurulent exudate	
Absent	**35 (38)**
Little	**35 (38)**
Much	**22 (24)**
Van der Heide score	
Low (0 – 6)	**50 (54)**
High (7 – 16)	**42 (46)**
Mayo score*	
0	**14 (16)**
1	**46 (52)**
2	**25 (29)**
3	**3 (3)**

*The Endoscopic Mayo score was available for 88 episodes (four episodes could not be classified according to the endoscopic Mayo score).

at presentation and endoscopic Mayo score (ρ 0.12; $P = 0.28$), van der Heide score (ρ 0.13; $P = 0.23$), or presence of ulcers (ρ 0.12; $P = 0.25$). Also, no correlation was found between the presence of abdominal pain and any endoscopic feature. A correlation was found between the presence of bloody stools and the endoscopic scores: Mayo ρ 0.35 ($P = 0.001$) and van der Heide ρ 0.43 ($P < 0.001$). There was no difference in the presence of ulcers in patients with grade 2 (37%) or grade 3 diarrhea (33%; $P = 0.73$). In 15 (24%) of the complete colonoscopies the ascending colon was more severely affected than the descending colon (an example can be seen in Figure 1e&f). Moreover, in 5 out of 64 colonoscopies (8%) endoscopic signs of inflammation were only seen in the ascending colon. Endoscopic features and their association with symptoms and treatment management have been summarized in Table 3.

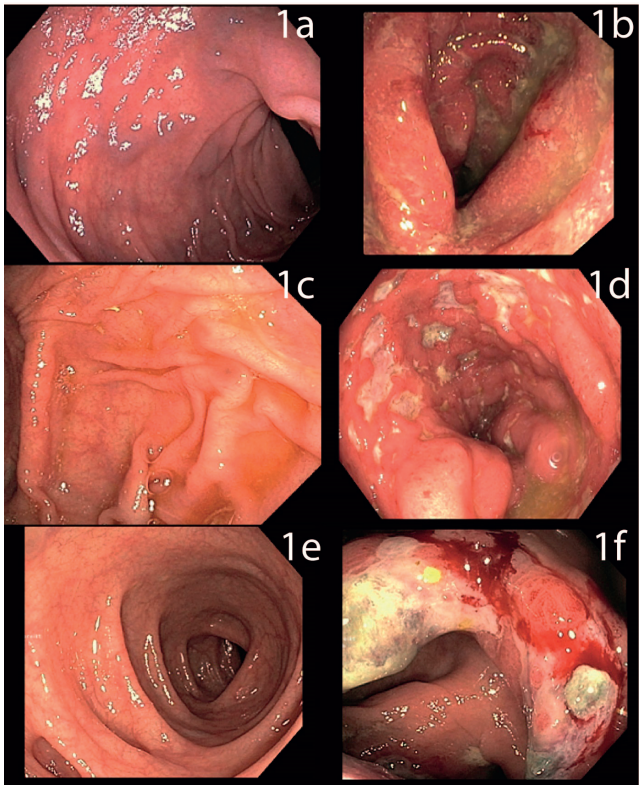

Figure 1a-1f. Examples of differences in immune-checkpoint inhibition-related colitis.
Figures 1a & 1b show two different patients with grade 2 diarrhea. Figure 1a shows no abnormalities on colonoscopy. Figure 1b shows a swollen, erosive and friable mucosa.
Figures 1c & 1d show two different patients with grade 3 diarrhea. Figure 1c shows no abnormalities on colonoscopy. Figure 1d shows a deeply red colon where the vascular pattern is partially absent, the mucosa appears severely friable and multiple ulcers can be seen.
Figures 1e & 1f show a single patient with grade 1 diarrhea. During colonoscopy the entire descending colon (1e) showed no abnormalties, while the ascending colon (1f) showed a swollen, severely friable mucosa, with deep ulcers.

Table 3. Endoscopic features and association with symptoms and treatment management in 92 episodes of diarrhea

	Total No. (%)	Grade of diarrhea† G2/G3 No. (%)	P value	Bloody stools no/yes No. (%)	P value	Need for infliximab no/yes No. (%)	P value
Endoscopic features							
Endoscopic Mayo*							
0-1 (low)	60 (68)	21 (44)/27 (56)	0.84	47 (78)/13 (22)	< 0.01	32 (53)/28 (47)	< 0.01
2-3 (high)	28 (32)	12 (46)/14 (54)		13 (46)/15 (54)		6 (21)/22 (79)	
Total van der Heide score							
0-6 (low)	50 (54)	20 (49)/21 (51)	0.53	44 (88)/6 (12)	< 0.01	29 (58)/21 (42)	< 0.01
7-12 (high)	42 (46)	15 (42)/21 (58)		19 (45)/23 (55)		12 (29)/30 (71)	
Ulcers							
No	63 (69)	22 (44)/28 (56)	0.73	47 (75)/16 (25)	0.06	35 (56)/28 (44)	< 0.01
Yes	29 (31)	13 (48)/14 (52)		16 (55)/13 (45)		6 (21)/23 (79)	
Pancolitis#							
No	20 (32)	7 (50)/7 (50)	0.36	17 (85)/3 (15)	0.13	15 (75)/5 (25)	< 0.01
Yes	42 (68)	14 (36)/25 (64)		28 (67)/14 (33)		10 (24)/32 (76)	

Cases with missing values not included in χ^2 test.

*The Endoscopic Mayo score was available for 88 episodes (four episodes could not be classified according to the endoscopic Mayo score).

#Pancolitis only available for 62 episodes in which a full colonoscopy was performed.

†Grade of diarrhea only G2 versus G3

Histological features

In 90 episodes (94%) biopsies were taken during endoscopy. Histopathological features have been summarized in Table 4. In the majority of episodes patients had received immunosuppressive drugs before the endoscopic procedure (52%, *N* = 47). Median days on high-dose steroids was 4 (IQR 2 – 6). The most common change was an increase in lamina propria cellularity (83%, *N* = 75), primarily consisting of mononuclear cells (Supplementary Figure 2). The second most common change was neutrophilic infiltration, either intraepithelial (79%, *N* = 71) or as crypt abscesses (62%, *N* = 56). In both circumstances, usually mild and patchy. Mild to prominent intraepithelial lymphocytosis was present in only 10% (*N* = 9). Small foci with minimal increase in intraepithelial lymphocytes were noted in an additional 15 cases (17%). Increased numbers of apoptotic cells were seen in the crypts in 42% (*N* = 38) but usually this was mild (*N* = 28). Outcomes of exploratory association analysis of histopathological features with various clinical and endoscopic features are displayed in Supplementary Table 3. Analogous to endoscopic features, none of the histopathological features had an association with the grade of diarrhea. However, multiple histopathological features were associated with endoscopic features (such as Mayo score and the presence of ulcers), bloody stools and the need for infliximab. Endoscopic and histopathological features had no association with different types of immune-

check point inhibitors (Supplementary Table 4). Also, histopathological features did not correlate with whether or not immunosuppressive therapy had been administered before taking the biopsies (data not shown).

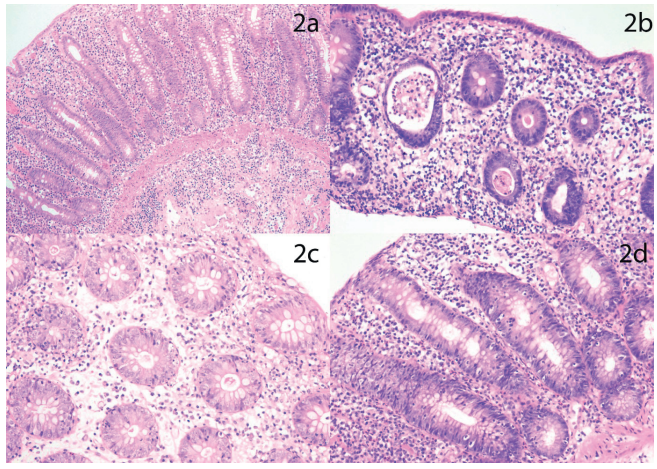

Figure 2. Representative HE sections demonstrating immune-checkpoint inhibition-related colitis
Representative HE sections demonstrating immune-checkpoint inhibition-related colitis characterized by increased lamina propria cellularity (2a, 2b and 2d). 2a, extension of the infiltrate into the submucosa. 2b, neutrophilic inflammation with a crypt abscesses, mild cryptitis, mucin depletion of epithelial cells and small foci with minimal increase in intraepithelial lymphocytes. 2c, apoptotic cells in crypt epithelium. 2d, prominent intraepithelial lymphocytosis.

Initial management of IRC

In all but 4 episodes patients received high-dose corticosteroids. Median time between onset of symptoms and start of high-dose corticosteroid therapy was 5 days (IQR 3 – 11). Of all 92 episodes of diarrhea treated with high-dose corticosteroids, 32 (35%) were initially treated with a dosage of < 1 mg/kg, 57 episodes (62%) with 1 mg/kg and 3 episodes (3%) with > 1 mg/kg. Patients used high-dose corticosteroids for a median of 44 days (IQR 29 – 73). In twelve episodes (13%) patients received budesonide as local treatment.

Steroid-refractory colitis

In 54 (56%) episodes patients required infliximab (5 mg/kg) for corticosteroid-refractory colitis, and of those patients 50% (N = 27) were given more than one cycle infliximab. Median time between start of prednisone and start of infliximab was 9 days (IQR 5 – 19.5) and median time to response on infliximab was 2 days (IQR 1 – 4). In three episodes patients required additional immunosuppressive agents such as mycophenolic acid or tacrolimus. None of 15 patients with grade 1 diarrhea required infliximab therapy. We did not see any difference in the requirement of infliximab for patients that presented with grade 2 (68%) or grade 3 (67%) diarrhea. Interestingly, in 79% of episodes in which ulcers were seen

Table 4. Histopathological features of biopsies taken in 90 endoscopies

Histopathological feature	No. (%)
Lamina propria cellularity	
Normal	**15 (17)**
Increased	
Focal	**7 (8)**
Patchy	**24 (27)**
Diffuse, superficial	**4 (4)**
Diffuse, transmucosal - mild	**24 (26)**
Diffuse, transmucosal - moderate	**16 (18)**
Diffuse, transmucosal – severe	**0 (0)**
Crypt architecture	
Normal	**58 (64)**
Irregular – mild	**23 (26)**
Irregular – moderate	**8 (9)**
Irregular – severe	**1 (1)**
Mucosal surface	
Flat/normal	**74 (82)**
Irregular	**15 (17)**
Villous	**1 (1)**
Apoptotic cells in crypt epithelium	
Absent/hardly any	**52 (58)**
Mild	**28 (31)**
Moderate	**6 (7)**
Severe	**4 (4)**
Extension of chronic inflammatory infiltrate into submucosa	
Not present	**46 (58)**
Present	**33 (42)**
Location of intraepithelial neutrophilic infiltration	
Absent	**19 (21)**
Present in crypt epithelium	**8 (9)**
Present in superficial epithelium	**16 (18)**
Present in crypt and superficial epithelium	**47 (52)**
Grade of intraepithelial neutrophilic infiltration	
None	**19 (21)**
Minimal	**15 (17)**
Mild	**46 (51)**
Moderate	**10 (11)**
Severe	**0 (0)**
Neutrophilic crypt abscesses	
Absent	**34 (38)**
Mild	**46 (51)**
Moderate	**8 (9)**
Severe	**2 (2)**
Location of intraepithelial lymphocytosis	
Absent	**66 (73)**
Present in crypt epithelium	**4 (5)**
Present in superficial epithelium	**8 (9)**
Present in crypt and superficial epithelium	**12 (13)**

Table 4. Histopathological features of biopsies taken in 90 endoscopies (*continued*)

Histopathological feature	No. (%)
Grade of intraepithelial lymphocytosis	
Absent	**66 (73)**
Minimal and patchy	**15 (17)**
Mild	**6 (7)**
Moderate	**2 (2)**
Severe	**1 (1)**
Mucin depletion of epithelial cells	
Not present	**46 (51)**
Mild	**32 (36)**
Moderate	**10 (11)**
Severe	**2 (2)**
Ulceration	
Absent	**71 (79)**
Present	**19 (21)**
Granuloma	
Absent	**85 (94)**
Present in lamina propria	**4 (5)**
Present in submucosa	**1 (1)**

during endoscopy, patients needed infliximab, while this was only the case for 44% of episodes in which no ulcers were seen (P = 0.002). Patients with a Van der Heide score between 7 and 12 (high score) received infliximab in 71% of episodes, while this was 42% in case of a Van der Heide score of 0–6 (low score; P = 0.005). Similarly, with these data, 79% of patients with a Mayo score of 2–3 (high score) received infliximab compared to 47% for patients with a Mayo score of 0–1 (low score; P = 0.005). Seventy-six percent of patients with a pancolitis (≥ 3 affected segments) required infliximab, while this was only the case in 25% of patients with < 3 affected segments (P < 0.001). In total six patients (16%) had a response (complete response (CR) or partial response (PR)) in the group that did not receive infliximab versus ten patients (22%) in the group that did receive infliximab (P = 0.53). Also when looking at disease control rate (stable disease + PR + CR) there was no significant difference in response in patients that received infliximab versus those that did not. Disease control rate was 59% (22 out of 37 patients) in the group of patients that did not receive infliximab versus 44% (20 out of 46 patients) in the group of patients that did receive infliximab (P = 0.15). No serious infliximab related side-effects were seen.

Colitis and best overall response

In total 60 patients with colitis and a cutaneous melanoma had an evaluable response. In the group of patients treated with anti-CTLA-4 monotherapy the response rate was 18% (7 out of 39 patients). In the group of patients treated with anti-PD1 monotherapy the response rate was 44% (4 out of 9 patients) and it was 33% (4 out of 12) for patients treated with the combination of anti-CLTA-4 and anti-PD1. These response rates appear

not different from what has been demonstrated in phase III clinical trials (Checkmate-069 and -067 or Keynote-006).

DISCUSSION

In this retrospective study on IRC we have shown that there is no significant difference between patients with grade 2 or 3 diarrhea with regard to endoscopic severity scores, histopathological features, the requirement for infliximab, or the presence of ulcers. This is important because IRC is usually managed based on the grade of diarrhea according to the CTCAE. Instead, we have found a correlation between endoscopic features and the need for immune suppression beyond high-dose corticosteroids. In that light our findings are relevant, as they would help the clinician to more rapidly intensify immune suppression, with the aim to reduce the time to recovery. Endoscopic characteristics in IRC are very diverse and there are no available validated scoring systems. Of note, due to the retrospective nature of our study the endoscopic findings may have influenced physicians in their choice for management. Based on our findings we suggest that these variables are taken into account in future scores for IRC. Our study suggests that the presence of ulcers and pancolitis (≥ 3 affected colon segments) are predictors of steroid-refractory colitis, perhaps warranting immediate start of infliximab upon colonoscopy.

Given the rapid improvement in symptoms after infliximab treatment (median time to response on infliximab was two days) we therefore strongly advise to consider the use of infliximab earlier, especially in patients with ulcerations or pancolitis. Furthermore, our study supports the findings by Schadendorf et al. who showed that infliximab did not seem to affect the development of a response or the durability of response [21]. The rationale of early initiation of infliximab is based on its efficacy in patients with inflammatory bowel disease (IBD). In IBD treatment with infliximab resulted in more clinical responses, mucosal healing, sparing of steroids, fewer admissions to the hospital and less surgical interventions [22, 23]. An earlier start of infliximab –top down approach– is now increasingly used in severe cases of IBD. Currently a trial is being performed that investigates early treatment with infliximab in immune-checkpoint inhibition induced colitis (NCT02763761).

In our study we have also shown that in 23% of colonoscopies the ascending colon was more severely affected than the descending colon. In these cases, the severity of IRC would have been underestimated by sigmoidoscopy only. Therefore, performing a full colonoscopy in patients that present with grade ≥ 2 diarrhea may have added value to sigmoidoscopy, as the underestimated amount and severity of colonic inflammation may

results in under treatment of patients. The choice for colonoscopy however, has to be judged in relation to other factors such as: burden for the patient and the possibility of perforation, which is about 2–4 times higher than that of sigmoidoscopy [24]. Therefore, if severe ulceration is present in the left-sided colon, assessment of the right-sided colon is not necessary for decisions on further management. CT-colonography could be used as an alternative to colonoscopy, as it has a slightly lower iatrogenic perforation rate. However, CT-colonography has low sensitivity for correct detection of acute colitis (64%), offers no possibility to take biopsies and still is a considerable burden for patients [24-26].

In this largest series to date analyzing histopathology of IRC we found that IRC is most typically described as an increase in lamina propria cellularity (83%), commonly extending slightly into the submucosa (42%), combined with patchy neutrophilic infiltrate (intraepithelial, 79% and/or crypt abscesses, 62%). In 36% of cases there were also some irregularities in the crypt architecture present but still the overall morphology appeared different from IBD. This is in line with the results of earlier analyses [20, 27]. Some cases showed an increase in intraepithelial lymphocytes and/or apoptosis but these features were inconsistent. Histopathological features neither correlate with the different types of immune-checkpoint inhibitors used nor with the time point of start of immunosuppression (before or after biopsies were taken). Many of the histopathological features were correlated with endoscopic features. Therefore, histopathology did not seem to have an added value to guide therapy beyond what was found endoscopically. Mucosal biopsies appear to mainly serve to confirm diagnosis.

Despite the information that is provided by colonoscopy and mucosal biopsies, which may help guide optimal management of diarrhea and colitis, one could argue that performing a colonoscopy will not change the management of IRC. Based on management algorithms patients will start with high-dose steroids and in case of insufficient improvement of symptoms, the addition of infliximab within several days – all without any guidance of information from colonoscopy or mucosal biopsies. In addition colonoscopies are cumbersome for patients, not without risk of perforation and the time benefit until infliximab treatment might be marginal with a waiting time of several days before a colonoscopy can be performed. Currently, it is not known whether omission of a colonoscopy affects the outcome of patients developing immunotherapy-induced gastrointestinal toxicity negatively, and therefore should be the subject of further studies. Nevertheless, patients with severe IRC should be treated promptly to prevent serious complications, such as perforation. On the other hand, patients with only mild or no active colitis, based on endoscopic findings, could be sufficiently treated with local steroids only.

Limitations of our study are the retrospective analysis, the fact that there might be classification bias due to the retrospective scoring of symptoms and endoscopic severity. Also, biopsies were taken in a non-standardized manner.

CONCLUSIONS

The correlation between grade of diarrhea and endoscopic features for severity of colitis is poor. Patients with higher endoscopic severity scores, ulcers, or a pancolitis needed the addition of infliximab more often. Therefore, endoscopy may have value in the evaluation of the severity of immune-checkpoint inhibitor-related colitis and may help in decision making for optimal management.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. Van der Heide score for the assessment of endoscopic activity of colitis

		Grade	
Endoscopic feature	**0**	**1**	**2**
Color	**Normal**	**Red**	**Deeply red**
Vascular patter	**Normal**	**Partially absent**	**Completely absent**
Friability	**Normal**	**Slightly friable**	**Severely friable**
Granularity	**Absent**	**Fine granularity**	**Coarse granularity**
Rectal valves	**Sharp**	**Swollen**	**Absent**
Ulcers	**Absent**	**Few**	**Multiple**
Spontaneous bleeding	**Absent**	**Discrete**	**Severe**
Mucopurulent exudate	**Absent**	**Little**	**Much**

A score of 0 – 6 is considered as low, while a score of 7 – 16 is considered as high

Endoscopic Mayo score for the assessment of endoscopic activity of colitis

Grade	Findings
0	**Normal mucosa**
1	**Mild: Erythema, decreased vascular pattern, mild friability**
2	**Moderate: marked erythema, absent vascular pattern, friability, erosions**
3	**Severe: spontaneous bleeding, ulcers**

Supplementary Table 2. Description of patients that underwent surgery due to bowel perforation as a complication of immune-related colitis

Patient	Tumor type	Treatment	Grade of diarrhea at presentation	Days between diarrhea and start immunosuppression	Immunosuppression	Days between diarrhea and endoscopy	Ulcers	Pancolitis	Mayo score	Addition of infliximab	Days between endoscopy and start infliximab	Days between endoscopy and surgery	Surgery
1	Uveal melanoma	Ipilimumab 10 mg/kg + RFA	2	13	Prednisone 1 mg/kg	14	yes	yes	2	yes	3	10	Left sided hemicolectomy with colostomy
2	Cutaneous melanoma	Ipilimumab 3 mg/kg	2	2	Prednisone <1 mg/kg	9	yes	yes	NA	yes	0	8	Subtotal colectomy with ileostomy
3	Cutaneous melanoma	Ipilimumab 3 mg/kg	3	14	Prednisone <1 mg/kg	14	yes	yes	2	yes	12	9	Sigmoid resection with colostomy

Abbreviations: NA; Not Available, RFA; radio-frequency ablation

Supplementary Table 3. Histopathological features and association with symptoms, endoscopic features and treatment management from 90 biopsies

Histopathological features	Total No. (%)	Grade of diarrhea† G2/ G3 No. (%)	P value	Bloody stools no/yes No. (%)	P value	Endoscopic Mayo score 0-1/2-3 No. (%)	P value	Need for infliximab no/yes No. (%)	P value
Lamina propria cellularity									
Normal	15 (16)	6 (50)/6 (50)	0.16	14 (93)/1 (7)	0.01	15 (100)/0 (0)	< 0.01	11 (73)/4 (27)	0.02
Increased									
Focal	7 (8)	3 (75)/1 (25)		6 (86)/1 (14)		7 (100)/0 (0)		5 (71)/2 (29)	
Patchy	24 (27)	8 (40)/12 (60)		19 (79)/5 (21)		14 (70)/6 (30)		8 (33)/16 (67)	
Diffuse, superficial	4 (4)	0 (0)/4 (100)		3 (75)/1 (25)		3 (75)/1 (25)		3 (75)/1 (25)	
Diffuse, transmucosal - mild	24 (27)	13 (59)/9 (41)		12 (50)/12 (50)		12 (50)/12 (50)		6 (25)/18 (75)	
Diffuse, transmucosal - moderate	16 (18)	4 (31)/9 (69)		7 (44)/9 (56)		7 (44)/9 (56)		7 (44)/9 (56)	
Diffuse, transmucosal – severe	0 (0)	0 (0)		0 (0)		0 (0)		0 (0)	
Crypt architecture									
Normal	58 (64)	21 (44)/27 (56)	0.73	48 (83)/10 (17)	< 0.001	46 (82)/10 (18)	< 0.001	30 (52)/28 (48)	0.26
Irregular – mild	23 (26)	9 (47)/10 (53)		12 (52)/11 (48)		10 (48)/11 (52)		7 (30)/16 (70)	
Irregular – moderate	8 (9)	4 (57)/3 (43)		1 (13)/7 (87)		2 (25)/6 (75)		3 (38)/5 (62)	
Irregular – severe	1 (1)	0 (0)/1 (100)		0 (0)/1 (100)		0 (0)/1 (100)		0 (0)/1 (100)	
Mucosal surface									
Flat/normal	74 (82)	29 (47)/33 (53)	0.38	56 (76)/18 (24)	< 0.001	54 (76)/17 (24)	< 0.001	34 (46)/40 (54)	0.61
Irregular	15 (17)	4 (33)/8 (67)		5 (33)/10 (67)		4 (29)/10 (71)		6 (40)/9 (60)	
Villous	1 (1)	1 (100)/0 (0)		0 (0)/1 (100)		0 (0)/1 (100)		0 (0)/1 (100)	
Apoptotic cells in crypt epithelium									
Absent/hardly any	52 (58)	17 (41)/25 (59)	0.75	39 (75)/13 (25)	0.27	37 (76)/12 (24)	0.30	30 (58)/22 (42)	0.03
Mild	28 (31)	13 (50)/13 (50)		15 (54)/13 (46)		16 (59)/11 (41)		7 (25)/21 (75)	
Moderate	6 (7)	2 (50)/2 (50)		4 (67)/2 (33)		3 (50)/3 (50)		2 (33)/4 (67)	
Severe	4 (4)	2 (67)/1 (33)		3 (75)/1 (25)		2 (50)/2 (50)		1 (25)/3 (75)	
Extension of chronic inflammatory infiltrate into submucosa									
Not present	46 (58)	16 (46)/19 (54)	0.73	36 (78)/10 (22)	0.001	39 (87)/6 (13)	< 0.001	24 (52)/22 (48)	< 0.01
Present	33 (42)	15 (48)/16 (52)		14 (42)/19 (58)		8 (26)/22 (74)		7 (21)/26 (79)	

Supplementary Table 3. Histopathological features and association with symptoms, endoscopic features and treatment management from 90 biopsies (*continued*)

Histopathological features	Total No. (%)	Grade of diarrhea† G2/ G3 No. (%)	P value	Bloody stools no/yes No. (%)	P value	Endoscopic Mayo score 0-1/2-3 No. (%)	P value	Need for infliximab no/yes No. (%)	P value
Location of intraepithelial neutrophilic infiltration									
Absent	19 (22)	6 (46)/7 (54)	0.79	17 (90)/2 (10)	0.03	17 (94)/1 (6)	0.03	16 (84)/3 (16)	< 0.001
Present in crypt epithelium	8 (9)	2 (29)/5 (71)		5 (63)/3 (37)		5 (63)/3 (37)		4 (50)/4 (50)	
Present in superficial epithelium	16 (18)	6 (43)/8 (57)		13 (81)/3 (19)		11 (73)/4 (27)		9 (56)/7 (44)	
Present in crypt and superficial epithelium	47 (52)	20 (49)/21 (51)		26 (55)/21 (45)		25 (56)/20 (44)		11 (23)/36 (77)	
Grade of intraepithelial neutrophilic infiltration									
None	19 (21)	6 (46)/7 (54)	0.59	17 (90)/2 (10)	< 0.01	17 (94)/1 (6)	< 0.001	16 (84)/3 (16)	< 0.001
Minimal	15 (17)	6 (55)/5 (45)		12 (80)/3 (20)		13 (93)/1 (7)		10 (67)/5 (33)	
Mild	46 (51)	17 (40)/26 (60)		29 (63)/17 (37)		25 (57)/19 (43)		11 (24)/35 (76)	
Moderate	10 (11)	5 (63)/3 (37)		3 (30)/7 (70)		3 (30)/7 (70)		3 (30)/7 (70)	
Severe	0 (0)	0 (0)		0 (0)		0 (0)		0 (0)	
Neutrophilic crypt abscesses									
Absent	34 (38)	12 (44)/15 (56)	0.64	26 (77)/8 (23)	0.36	25 (76)/8 (24)	0.47	22 (65)/12 (35)	0.01
Mild	46 (51)	19 (50)/19 (50)		28 (61)/18 (39)		28 (65)/15 (35)		16 (35)/30 (65)	
Moderate	8 (9)	2 (25)/6 (75)		5 (63)/3 (37)		4 (50)/4 (50)		2 (25)/6 (75)	
Severe	2 (2)	1 (50)/1 (50)		2 (100)/0 (0)		1 (50)/1 (50)		0 (0)/2 (100)	
Location of intraepithelial lymphocytosis									
Absent	66 (73)	25 (42)/34 (58)	0.27	42 (64)/24 (36)	0.55	39 (62)/24 (38)	0.32	27 (41)/39 (59)	0.70
Present in crypt epithelium	4 (5)	3 (100)/0 (0)		3 (75)/1 (25)		3 (75)/1 (25)		2 (50)/2 (50)	
Present in superficial epithelium	8 (9)	3 (43)/4 (57)		6 (75)/2 (25)		7 (88)/1 (12)		4 (50)/4 (50)	
Present in crypt and superficial epithelium	12 (13)	3 (50)/3 (50)		10 (83)/2 (17)		9 (82)/2 (18)		7 (58)/5 (42)	
Grade of intraepithelial lymphocytosis									
Absent	66 (73)	25 (42)/34 (58)	0.59	42 (64)/24 (36)	0.59	39 (62)/24 (38)	0.43	27 (41)/39 (59)	0.38
Minimal and patchy	15 (17)	7 (58)/5 (42)		11 (73)/4 (27)		11 (79)/3 (21)		7 (47)/8 (53)	
Mild	6 (7)	2 (50)/2 (50)		5 (83)/1 (17)		5 (83)/1 (17)		3 (50)/3 (50)	
Moderate	2 (2)	0 (0)/0 (0)		2 (100)/0 (0)		2 (100)/0 (0)		2 (100)/0 (0)	
Severe	1 (1)	0 (0)/0 (0)		1 (100)/0 (0)		1 (100)/0 (0)		1 (100)/0 (0)	

Supplementary Table 3. Histopathological features and association with symptoms, endoscopic features and treatment management from 90 biopsies (*continued*)

Histopathological features	Total No. (%)	Grade of diarrhea† G2/ G3 No. (%)	P value	Bloody stools no/yes No. (%)	P value	Endoscopic Mayo score 0-1/2-3 No. (%)	P value	Need for infliximab no/yes No. (%)	P value
Mucin depletion of epithelial cells									
Not present	46 (51)	15 (41)/22 (59)	0.37	37 (80)/9 (20)	< 0.001	38 (84)/7 (16)	< 0.001	24 (52)/22 (48)	0.29
Mild	32 (36)	15 (56)/12 (44)		22 (69)/10 (31)		19 (66)/10 (34)		13 (41)/19 (59)	
Moderate	10 (11)	4 (44)/5 (56)		1 (10)/9 (90)		1 (10)/9 (90)		3 (30)/7 (70)	
Severe	2 (2)	0 (0)/2 (100)		1 (50)/1 (50)		0 (0)/2 (100)		0 (0)/2 (100)	
Ulceration									
Absent	71 (79)	27 (47)/30 (53)	0.53	48 (68)/23 (32)	0.95	55 (78)/16 (22)	< 0.001	35 (49)/36 (51)	0.07
Present	19 (21)	7 (39)/11 (61)		13 (68)/6 (32)		3 (20)/12 (80)		5 (26)/14 (74)	
Granuloma									
Absent	85 (94)	31 (44)/40 (56)	0.22	58 (68)/27 (32)	0.33	57 (70)/25 (30)	0.06	38 (45)/47 (55)	0.39
Present in lamina propria	4 (5)	3 (75)/1 (25)		3 (75)/1 (25)		1 (25)/3 (75)		1 (25)/3 (75)	
Present in submucosa	1 (1)	0 (0)/0 (0)		0 (0)/1 (100)		0 (0)		1 (100)/0 (0)	

Cases with missing values not included in χ^2 test

†Grade of diarrhea only G2 versus G3

*The Endoscopic Mayo score was available for 86 episodes (four episodes could not be classified according to the endoscopic Mayo score).

Supplementary Table 4. Association of different immunotherapies with histopathological and endoscopic features from 92 endoscopies

	Total No. (%)	Anti CTLA-4 No. (%)	Anti PD-1 No. (%)	Combined anti CTLA-4 + anti PD-(L)1 No. (%)	Sequential anti CTLA-4 + anti PD-1 No. (%)	P value
Lamina propria cellularity						
Normal	15 (16)	4 (8)	5 (25)	4 (33)	2 (24)	0.12
Increased						
Focal	7 (8)	3 (6)	4 (20)	0 (0)	0 (0)	
Patchy	24 (27)	11 (22)	4 (20)	5 (42)	4 (50)	
Diffuse, superficial	4 (4)	3 (6)	1 (5)	0 (0)	0 (0)	
Diffuse, transmucosal - mild	24 (27)	17 (34)	3 (15)	3 (25)	1 (13)	
Diffuse, transmucosal - moderate	16 (18)	12 (24)	3 (15)	0 (0)	1 (13)	
Diffuse, transmucosal – severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Crypt architecture						
Normal	58 (64)	29 (58)	15 (75)	9 (75)	5 (63)	0.24
Irregular – mild	23 (26)	18 (36)	1 (5)	2 (17)	2 (25)	
Irregular – moderate	8 (9)	3 (6)	3 (15)	1 (8)	1 (12)	
Irregular – severe	1 (1)	0 (0)	1 (5)	0 (0)	0 (0)	
Mucosal surface						
Flat/normal	74 (82)	44 (88)	16 (80)	9 (75)	5 (63)	0.28
Irregular	15 (17)	6 (12)	3 (15)	3 (25)	3 (37)	
Villous	1 (1)	0 (0)	1 (5)	0 (0)	0 (0)	
Apoptotic cells in crypt epithelium						
Absent/hardly any	52 (58)	29 (58)	11 (55)	8 (67)	4 (50)	0.72
Mild	28 (31)	14 (28)	8 (40)	2 (17)	4 (50)	
Moderate	6 (7)	5 (10)	0 (0)	1 (8)	0 (0)	
Severe	4 (4)	2 (4)	1 (5)	1 (8)	0 (0)	
Extension of chronic inflammatory infiltrate into submucosa1						
Not present	46 (58)	25 (53)	13 (81)	6 (60)	2 (33)	0.15
Present	33 (42)	22 (47)	3 (19)	4 (40)	4 (67)	

Supplementary Table 4. Association of different immunotherapies with histopathological and endoscopic features from 92 endoscopies (*continued*)

	Total No. (%)	Anti CTLA-4 No. (%)	Anti PD-1 No. (%)	Combined anti CTLA-4 + anti PD-(L)1 No. (%)	Sequential anti CTLA-4 + anti PD-1 No. (%)	P value
Location of intraepithelial neutrophilic infiltration						
Absent	19 (21)	7 (14)	6 (30)	4 (34)	2 (25)	0.68
Present in crypt epithelium	8 (9)	5 (10)	2 (10)	1 (8)	0 (0)	
Present in superficial epithelium	16 (18)	9 (18)	5 (25)	1 (8)	1 (13)	
Present in crypt and superficial epithelium	47 (52)	29 (58)	7 (35)	6 (50)	5 (62)	
Grade of intraepithelial neutrophilic infiltration						
None	19 (21)	7 (14)	6 (30)	4 (33)	2 (25)	0.63
Minimal	15 (17)	9 (18)	4 (20)	2 (17)	0 (0)	
Mild	46 (51)	29 (58)	8 (40)	5 (42)	4 (50)	
Moderate	10 (11)	5 (10)	2 (10)	1 (8)	2 (25)	
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Neutrophilic crypt abscesses						
Absent	34 (38)	16 (32)	11 (55)	3 (25)	4 (50)	0.49
Mild	46 (51)	27 (54)	7 (35)	9 (75)	3 (38)	
Moderate	8 (9)	5 (10)	2 (10)	0 (0)	1 (12)	
Severe	2 (2)	2 (4)	0 (0)	0 (0)	0 (0)	
Location of intraepithelial lymphocytosis						
Absent	66 (74)	37 (74)	11 (55)	10 (84)	8 (100)	0.08
Present in crypt epithelium	4 (4)	3 (6)	0 (0)	1 (8)	0 (0)	
Present in superficial epithelium	8 (9)	2 (4)	5 (25)	1 (8)	0 (0)	
Present in crypt and superficial epithelium	12 (13)	8 (16)	4 (20)	0 (0)	0 (0)	
Grade of intraepithelial lymphocytosis						
Absent	66 (73)	37 (74)	11 (55)	10 (84)	8 (100)	0.16
Minimal and patchy	15 (17)	10 (20)	4 (20)	1 (8)	0 (0)	
Mild	6 (7)	1 (2)	4 (20)	1 (8)	0 (0)	
Moderate	2 (2)	2 (4)	0 (0)	0 (0)	0 (0)	
Severe	1 (1)	0 (0)	1 (5)	0 (0)	0 (0)	

Supplementary Table 4. Association of different immunotherapies with histopathological and endoscopic features from 92 endoscopies (*continued*)

	Total No. (%)	Anti CTLA-4 No. (%)	Anti PD-1 No. (%)	Combined anti CTLA-4 + anti PD-(L)1 No. (%)	Sequential anti CTLA-4 + anti PD-1 No. (%)	P value
Mucin depletion of epithelial cells						
Not present	46 (51)	22 (44)	14 (70)	6 (50)	4 (50)	0.65
Mild	32 (36)	22 (44)	3 (15)	4 (33)	3 (38)	
Moderate	10 (11)	5 (10)	2 (10)	2 (17)	1 (12)	
Severe	2 (2)	1 (2)	1 (5)	0 (0	0 (0)	
Ulceration						
Absent	71 (79)	40 (80)	17 (85)	9 (75)	5 (63)	0.60
Present	19 (21)	10 (20)	3 (15)	3 (25)	3 (37)	
Granuloma						
Absent	85 (94)	47 (94)	20 (100)	10 (84)	8 (100)	0.19
Present in lamina propria	4 (5)	3 (6)	0 (0)	1 (8)	0 (0)	
Present in submucosa	1 (1)	0 (0)	0 (0)	1 (8)	0 (0)	
Endoscopic Mayo*						
0-1 (low)	60 (68)	34 (57)	16 (84)	7 (64)	3 (43)	0.21
2-3 (high)	28 (32)	17 (33)	3 (16)	4 (36)	4 (57)	
Total van der Heide score						
0-6 (low)	50 (54)	25 (48)	16 (80)	6 (50)	3 (37)	0.07
7-12 (high)	42 (46)	27 (52)	4 (20)	6 (50)	5 (63)	

Cases with missing values not included in χ^2 test

*The Endoscopic Mayo score was available for 88 episodes (four episodes could not be classified according to the endoscopic Mayo score).

Chapter 4

Clinical and radiological response of BRAF-inhibition and MEK-inhibition in patients with brain metastases from BRAF-mutated melanoma

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ABSTRACT

Background

Patients with brain metastases (BM) from melanoma have an overall survival of 2 - 6 months after whole brain radiotherapy. Targeted therapy (TT) is an effective treatment for BRAF-mutated metastatic melanoma. Moreover, recent studies indicate intracranial responses of TT in patients with BM.

Methods

We analysed 146 patients with BM from BRAF-mutated melanoma treated with vemurafenib, dabrafenib, or dabrafenib + trametinib between 2010 and 2016. We determined clinical and radiological response, progression-free survival (PFS) and overall survival (OS).

Results

Median OS of patients treated with dabrafenib + trametinib was 11.2 months (n = 30; 95% CI, 6.8 – 15.7), 8.8 months for dabrafenib alone (n = 31; 95% CI, 3.9 – 13.7) and 5.7 months for vemurafenib (n = 85; 95% CI, 4.6 – 6.8). A significantly longer OS was observed in the dabrafenib + trametinib group than in the vemurafenib group (HR for death, 0.52; 95% CI, 0.30 – 0.89; p = 0.02). Median intracranial PFS of all patients was 4.1 months. Median intracranial PFS for patients treated with dabrafenib + trametinib was 5.8 months (95% CI, 3.2 – 8.5), 5.7 months (95% CI, 3.0 – 8.4) for dabrafenib and 3.6 months (95% CI, 3.5 – 3.8) for vemurafenib (p = 0.54). Sixty-three patients (43%) had symptomatic BM. Intracranial disease control-rate at 8 weeks in these patients was 65% versus 70% extracranially. Neurological symptoms improved in 46% of patients with symptomatic BM, in 21% they remained stable.

Conclusions

Median OS in patients with BM from BRAF-mutated melanoma treated with dabrafenib + trametinib was significantly longer than for vemurafenib. Improvement of neurological symptoms was seen in almost half of symptomatic BM patients treated with TT.

INTRODUCTION

The incidence of metastatic melanoma has steadily increased over the past decades [1]. The incidence of brain metastases (BM) in patients with melanoma ranges from 10 to 73% based on clinical and post-mortem series [2-7]. Brain metastases from malignant melanoma carry a poor prognosis with a median survival of less than six months [8]. Before 2011, therapeutic options for BM from melanoma were local therapy such as surgery and/or cranial radiotherapy (RT) and sometimes systemic chemotherapy. Since 2011 antibodies against CTLA-4 (ipilimumab) and antibodies against Programmed Death Cell Receptor-1 (PD-1; nivolumab and pembrolizumab) were approved for treatment of metastatic melanoma. Moreover, 40-60% of cutaneous melanoma have a mutation in the gene encoding *BRAF*, which leads to constitutive activation of downstream signalling through the mitogen-activated protein kinase (MAPK) pathway [9, 10]. Vemurafenib and dabrafenib are potent inhibitors of the mutated BRAF-protein. Both have shown to improve progression-free survival (PFS) and overall survival (OS) when compared to the chemotherapeutic dacarbazine in randomized phase 3 trials [11, 12]. The combination of BRAF inhibitors (BRAFi) and MEK inhibitors (MEKi) (e.g. vemurafenib + cobimetinib or dabrafenib + trametinib) has shown to improve OS even further [13-15]. In prospective studies, BRAFi showed intracranial responses in both patients with asymptomatic and symptomatic BM (sBM) from BRAF mutated melanoma ranging from 31 – 40% with a duration of 4 – 7 months [16, 17]. The effect of the combination of BRAFi and MEKi in melanoma patients with BM has recently been described by Davies et al. [18]. In this prospective phase 2 study the effect of dabrafenib + trametinib in four different patient cohorts with BM from melanoma (based on mutation status (BRAFV600E versus BRAFV600D/K/R), previous local brain therapy and symptoms of BM)) was evaluated. Dabrafenib + trametinib was active in all four groups with intracranial response rates ranging from 44 to 59%. The aim of our observational study is to compare radiological response, neurological benefit, PFS and OS of BRAFi as monotherapy, or in combination with a MEKi in patients with BRAF-mutated melanoma BM.

METHODS

Patient inclusion criteria

Patients included in the current study are patients with metastatic melanoma and newly diagnosed or progressive BM treated at the Netherlands Cancer Institute. All patients had stage IV melanoma that tested positive for a mutation in the *BRAF* gene (i.e. V600E, V600K). For response analysis, patients were categorized into three groups (vemurafenib, dabrafenib or dabrafenib + trametinib). Patients that switched from one targeted therapy

(TT) to another TT were placed in the group of the drug that they were taking during CT thorax/abdomen and MRI brain, if they had used that (combination of) drug(s) for more than 50% of the time. All patients were discussed in a multidisciplinary meeting prior to TT start.

Treatment

Patients received treatment in standard dosages: vemurafenib 960 mg b.i.d., dabrafenib 150 mg b.i.d and trametinib 2 mg q.d.. One cycle equals four weeks of treatment. Patients visited the outpatient clinic every four weeks for physical examination and blood sampling. Every eight weeks extracranial disease was assessed by CT scans of thorax and abdomen and intracranial disease by MRI of the brain. LDH and S100 serum levels were measured at baseline, at a maximum of 28 days before starting TT.

Response

Extracranial response was determined by RECIST 1.1. For intracranial response we used a modified RECIST 1.1, which allowed us to include BM ≥ 5 mm. Assessment of both extra- and intracranial response was done by a (neuro-)radiologist. Intracranial DCR was defined as stable disease (SD) + partial response (PR) + complete response (CR) and was measured at 8 weeks after treatment start and every 8 weeks thereafter. Clinical response was determined by retrospective analysis of the neurological symptoms in the electronic patient records. Neurological symptoms (i.e. headache, nausea, vomiting, cognitive function disorder, ataxia and seizures) were scored before treatment and every four weeks after treatment. They were classified as worsened, stable or improved. Symptomatic patients were patients that had at least one neurological symptom. Progression-free survival was measured from the date of treatment start until progression of disease (PD) as measured by contrast-enhanced CT thorax/abdomen and contrast-enhanced MRI brain, date of last known follow-up, death, or switch of therapy. Overall survival was measured from the date of treatment start until death by any cause, or date of last known follow-up.

Statistics

Kaplan-Meier curves were used to determine the median OS, median intracranial PFS and extracranial PFS. Log-rank, univariate and multivariate Cox regression analysis was used to assess prognostic factors for survival. Data were analyzed using SPSS Statistics software (IBM version 22).

RESULTS

Patient and treatment characteristics

Hundred forty-six patients with BM from BRAF mutated melanoma were treated with TT between January 2010 and March 2016. Median age was 54 years (range 23 – 80 years) and fifty-five percent of patients was male (n = 80). Melanoma BRAF mutation status was V600E in 129 patients (88%), V600K in 12 patients (8%), V600R in 2 patients (1%) and K601E, L579R and $V600_{unknown}$ in 1 patient each. Median time from diagnosis of the primary melanoma till BM was 39.4 months (range 0 – 373 months). Thirty-two patients (22%) received systemic therapy (for example DTIC or ipilimumab) for extracranial metastases, but none had been treated with TT. At study start, BM were either newly diagnosed (n = 130, 89%), or TT was given for progressive BM (n = 16, 11%). In 74% of patients TT was given as sole treatment and in 26% as adjuvant treatment directly after radiotherapy. Eleven patients (8%) had intracranial surgery for BM, with start of TT post-surgery for remaining BM. Forty-nine patients (39%) received RT before start of TT: WBRT (n = 33, 67%), stereotactic RT (n = 13, 27%), or both (n = 3, 6%). Twelve patients had a switch in TT during treatment: 11 cases due to toxicities and one patient because trametinib became available. Patient and treatment characteristics are summarized in Table 1. No significant differences in characteristics were seen between the 3 treatment groups.

Treatment during and after TT

During TT 44 patients (30%) received RT due to progression of BM: twenty-six patients (59%) WBRT and 18 patients stereotactic RT (41%). Twenty-three patients (52%) continued TT as treatment beyond progression. Thirty-eight (26%) patients received systemic therapy after PD on TT, which was immunotherapy in 95% of patients.

Intracranial and extracranial disease control rate

The mean number of cycles of TT was 6 (range 1 – 34). Intracranial DCR at 8 weeks after treatment start of all patients was 68% (37% SD, PR 26% and CR 5%) whereas extracranial DCR was 74% (32% SD, 40% PR and 2% CR). Intracranial DCR in both sBM and asymptomatic BM patients was borderline significantly lower than the extracranial DCR in both groups (sBM: intracranial 65% versus extracranial 70%; p = 0.04, asymptomatic BM: intracranial 70% versus extracranial 77%; p = 0.04; Table 2). Intracranial DCR was 81% (16/42 SD and 18/42 PR) in the group of patients that received prior local RT, compared to 73% (38/89 SD, 20/89 PR, 7/89 CR) in the group of patients that did not receive prior local RT (p = 0.04). There was no statistically significant difference in intracranial DCR in patients that received RT during TT (67%; 17/43 SD, 11/43 PR and 1/43 CR) versus those that did not (80%; 37/88 SD, 27/88 PR and 6/87 CR; p = 0.37).

Table 1. Baseline characteristics of patients with brain metastases from BRAF-mutated malignant melanoma

Characteristics	vemurafenib *n* = 85	dabrafenib *n* = 31	dabrafenib + trametinib *n* = 30	Total *n* = 146	*p* value
Age, years					0.15
Median (range)	53 (23-80)	52 (29-78)	58 (37-80)	54 (23-80)	
Gender *n* (%)					0.17
Male	43 (51)	16 (52)	21 (70)	80 (55)	
Female	42 (49)	15 (48)	9 (30)	66 (45)	
WHO performance status † n (%)					0.53
0	36 (42)	13 (42)	16 (53)	65 (45)	
1	29 (34)	13 (42)	11 (37)	53 (36)	
2	14 (17)	5 (16)	2 (7)	21 (14)	
3	6 (7)	0 (0)	1 (3)	7 (5)	
Lactate dehydrogenase (%)					0.39
< ULN	31 (36)	13 (42)	15 (50)	59 (40)	
> ULN	49 (58)	17 (55)	13 (43)	79 (54)	
Unknown	5 (6)	1 (3)	2 (7)	8 (6)	
S100B *n* (%)					0.87
≤ ULN	14 (16)	6 (19)	6 (20)	26 (18)	
> ULN	66 (78)	23 (74)	22 (73)	111 (76)	
Unknown	5 (6)	2 (7)	2 (7)	9 (6)	
Brain metastases ≥ 2 cm *n* (%)					0.42
Yes	30 (35)	7 (23)	9 (30)	46 (32)	
No	55 (65)	24 (77)	21 (70)	100 (68)	
Number of brain metastases *n* (%)					0.86
Single	20 (23)	9 (29)	9 (30)	38 (26)	
2-5	32 (38)	9 (29)	11 (37)	52 (36)	
> 5	33 (39)	13 (42)	10 (33)	56 (38)	
Symptoms of brain metastases *n* (%)					0.06
Symptomatic	34 (40)	19 (61)	10 (33)	63 (43)	
Asymptomatic	51 (60)	12 (39)	20 (67)	83 (57)	
Symptomatic BM patients dependent of corticosteroids *n* (%)					0.89
Yes	21 (62)	12 (63)	7 (70)	40 (64)	
No	13 (38)	7 (37)	3 (30)	23 (36)	
Radiotherapy during TT *n* (%)					0.96
None	60 (71)	21 (68)	21 (70)	102 (70)	
Stereotactic radiotherapy	7 (8)	7 (22)	4 (13)	18 (12)	
Whole brain radiotherapy	18 (21)	3 (10)	5 (17)	26 (18)	
Surgery of brain metastases *n* (%)					0.48
Yes	9 (11)	3 (10)	1 (3)	13 (9)	
No	76 (89)	28 (90)	29 (97)	133 (91)	
Treatment after progression on TT					0.07
Yes	19 (22)	13 (42)	6 (20)	38 (26)	
Anti-CTLA-4 monotherapy	10 (53)	3 (23)	2 (33)	15 (39)	
Anti-PD1 monotherapy	1 (5)	7 (54)	4 (66)	12 (32)	
Anti-CTLA-4 and subsequent anti-PD1	5 (26)	1 (8)	0 (0)	6 (16)	
Concurrent anti CTLA-4 and anti-PD1	1 (5)	2 (15)	0 (0)	3 (8)	
Temozolomide	2 (11)	0 (0)	0 (0)	2 (5)	
No	66 (78)	18 (58)	24 (80)	108 (74)	

† The World Health Organization (WHO) performance status of 0 indicates that the patient is asymptomatic and fully active, 1: the patient is restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, 2: ambulatory and capable of all self-care but unable to carry out any work activities and 3: > 50% in bed, but not bed bound. Capable of only limited self-care, confined to bed or chair 50% or more of waking hours.
TT; targeted therapy, ULN; upper limit of normal

Table 2. Disease control rate, progression-free survival, clinical response rate and overall survival in patients with BM from BRAF-mutated malignant melanoma treated with targeted therapy

	vemurafenib *n* = 85	dabrafenib *n* = 31	dabrafenib + trametinib *n* = 30	Total *n* = 146	*p* value
Intracranial response *n* (%)					0.60
CR	3 (3)	1 (3)	3 (10)	7 (5)	
PR	23 (27)	5 (16)	10 (33)	38 (26)	
SD	34 (40)	15 (48)	5 (17)	54 (37)	
PD	16 (19)	7 (23)	9 (30)	32 (22)	
NE	9 (11)	3 (10)	3 (10)	15 (10)	
Intracranial DCR	60 (71)	21 (68)	18 (60)	99 (68)	
Extracranial response *n* (%)					0.38
CR	3 (3)	0 (0)	0 (0)	3 (2)	
PR	34 (40)	11 (35)	13 (43)	58 (40)	
SD	27 (32)	12 (39)	8 (27)	47 (32)	
PD	5 (6)	3 (10)	5 (17)	13 (9)	
NE	16 (19)	5 (16)	4 (13)	25 (17)	
Extracranial DCR	64 (75)	23 (74)	21 (70)	108 (74)	
Intracranial PFS months (95% CI)	3.6 (3.5 – 3.8)	5.7 (3.0 – 8.4)	5.8 (3.2 – 8.5)	4.1 (3.2 – 5.0)	0.54
Extracranial PFS months (95% CI)	4.0 (3.3 – 4.7)	5.8 (3.3 – 8.3)	7.3 (3.9 – 10.8)	4.6 (3.4 – 5.9)	0.20
Clinical intracranial response *n* (%)					0.32
Improved	11 (32)	12 (63)	6 (60)	29 (46)	
Stable	8 (24)	4 (21)	1 (10)	13 (21)	
Worsened	12 (35)	2 (11)	2 (20)	16 (25)	
NE	3 (9)	1 (5)	1 (10)	5 (8)	
Overall survival months (95% CI)	5.7 (4.6 – 6.8)	8.8 (3.9 – 13.7)	11.2 (6.8 – 15.7)	6.6 (5.7 – 7.4)	**0.04**

CI; confidence interval, CR; complete response, DCR; disease control rate, NE; not evaluable, PD; progressive disease, PFS; progression-free survival, PR; partial response, SD; stable disease, ULN; upper limit of normal.

Clinical-neurological response

In 29 of 63 sBM patients (46%) neurological symptoms improved after TT; in 13 patients (21%) neurological symptoms remained stable and in 16 patients (25%) symptoms worsened during treatment. Five patients with sBM (8%) were not evaluable. Eleven of 34 sBM patients (32%) treated with vemurafenib showed improvement of neurological symptoms, while this was the case for 12 of 19 patients (63%) treated with dabrafenib and 6 of 10 patients (60%) treated with the combination of dabrafenib + trametinib. Forty-five percent of patients with sBM that used dexamethasone to alleviate neurological symptoms before TT could stop dexamethasone after TT. In the group of patients that had not received prior local RT before TT clinical neurological benefit was 84% (6/31 stable and 20/31 improved), while this was 59% (7/27 stable and 9/27 improved) in the patient group that had received prior local RT (p= 0.04). No statistical difference was noted in clinical neurological benefit for patients receiving RT during TT (71%; 5/14 stable and 5/14 improved) and patients that did not (73%; 8/44 stable and 24/44 improved, p = 0.33).

Intracranial progression free survival

The median intracranial PFS of all patients was 4.1 months (95% CI, 3.2 – 5.0). Median intracranial PFS for vemurafenib was 3.6 months (95% CI, 3.5 – 3.8), for dabrafenib 5.7 months (95% CI, 3.0 – 8.4) and for the combination of dabrafenib + trametinib 5.8 months (95% CI, 3.2 – 8.5). No significant difference in intracranial PFS was observed between dabrafenib + trametinib and vemurafenib (HR for disease progression 1.23; 95% CI, 0.77 – 1.96), nor was there a significant difference in intracranial PFS between dabrafenib + trametinib versus dabrafenib (HR for disease progression 1.05; 95% CI, 0.56 – 1.97). Median intracranial PFS in patients with SD (n = 54) was not significantly different from patients

with PR or CR (n = 45); 5.5 months (95% CI, 4.1 – 6.8) versus 6.1 months (95% CI, 5.1 – 7.2; p = 0.11). Radiotherapy prior to TT did not significantly impact intracranial PFS (4.3 months with prior RT) versus 4.1 months without (p = 0.47). Radiotherapy during TT did also not significantly influence intracranial PFS (4.8 months with RT during TT versus 4.1 months without RT; p = 0.51). A normal serum S100B level and no use of dexamethasone during TT were significant favourable prognostic factors for intracranial PFS in univariate Cox regression analysis. In multivariate Cox regression analysis a normal serum S100B level remained a significant favourable prognostic factor for intracranial PFS (HR 3.1; 95% CI, 1.6 – 6.1; p < 0.01; Table 3).

Table 3. Univariate and multivariate Cox regression analysis for intracranial progression-free survival

				Univariate analysis			Multivariate analysis	
Parameter	Total *n*	Categories	*n* (%)	Median intracranial PFS (months)	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Treatment	146	vemurafenib	85 (31)	3.6	1		1	
		dabrafenib	30 (58)	5.7	0.8 (0.5 – 1.3)	0.38	0.7 (0.4 – 1.2)	0.15
		dabrafenib + trametinib	21 (21)	5.8	0.8 (0.5 – 1.3)	0.39	1.0 (0.6 – 1.6)	0.93
WHO performance status†	146	0-1	118 (28)	4.4	1		1	
		2-3	81 (19)	3.2	1.4 (0.9 – 2.3)	0.19	1.5 (0.9 – 2.7)	0.14
Lactate dehydrogenase	138	≤ ULN	59 (79)	5.4	1		1	
		> ULN	43 (57)	3.6	1.4 (1.0 – 2.1)	0.07	1.1 (0.7 – 1.7)	0.67
S100B	137	≤ ULN	26 (111)	11.3	1		1	
		> ULN	19 (81)	3.6	2.3 (1.4 – 3.9)	**< 0.01**	3.1 (1.6 – 6.1)	**< 0.01**
Brain metastases ≥ 2 cm	146	No	100 (46)	4.4	1		1	
		Yes	69 (31)	3.6	1.4 (0.9 – 2.0)	0.10	1.2 (0.8 – 1.9)	0.38
Number of brain metastases	146	≤ 5	90 (56)	4.7	1		1	
		> 5	62 (38)	3.6	1.3 (0.9 – 2.0)	0.13	1.4 (0.9 – 2.2)	0.11
Symptoms of brain metastases	146	Asymptomatic	83 (63)	5.1	1		1	
		Symptomatic	57 (43)	3.7	1.3 (0.9 – 1.9)	0.14	1.1 (0.7 – 1.7)	0.73
Radiotherapy during TT	146	No	102 (44)	4.1	1.1 (0.8 – 1.7)	0.51	0.7 (0.4 – 1.1)	0.14
		Yes	70 (30)	4.8	1		1	
Dexamethasone during TT	146	No	52 (94)	5.8	1		1	
		Yes	36 (64)	3.6	1.6 (1.1 – 2.4)	**0.01**	1.4 (0.9 – 2.2)	0.15

† The World Health Organization (WHO) performance status of 0 indicates that the patient is asymptomatic and fully active, 1 that the patient is restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, 2 ambulatory and capable of all self-care but unable to carry out any work activities and 3 > 50% in bed, but not bed bound. Capable of only limited self-care, confined to bed or chair 50% or more of waking hours.
CI; confidence interval, HR; hazard ratio, OS; overall survival, TT; targeted therapy, ULN; upper limit of normal

Extracranial progression free survival

The median extracranial PFS for all patients was 4.6 months (95% CI, 3.4 – 5.9). Median extracranial PFS for vemurafenib was 4.0 months (95% CI, 3.3 – 4.7), for dabrafenib 5.8 months (95% CI, 3.3 – 8.3) and for the combination of dabrafenib + trametinib 7.3 months (95% CI, 3.9 – 10.8). No significant difference in extracranial PFS was observed between dabrafenib + trametinib and vemurafenib (HR 1.5; 95% CI, 0.95 – 2.50), nor was there a significant difference in extracranial PFS between dabrafenib + trametinib and dabrafenib (HR 1.71; 95% CI, 0.88 – 3.31). A normal serum S100B level, a normal serum LDH level, ≤ 5 BM and RT during TT were favourable prognostic factors for extracranial PFS in univariate Cox regression analysis. In multivariate Cox regression analysis a normal serum S100B level remained an independent favorable prognostic factor (Table 4).

Table 4. Univariate and multivariate Cox regression analysis for extracranial progression-free survival

				Univariate analysis			Multivariate analysis	
Parameter	Total *n*	Categories	*n* (%)	Median extracranial PFS (months)	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Treatment	146	vemurafenib	85 (31)	4.0	1		1	
		dabrafenib	30 (58)	5.8	1.0 (0.6 – 1.6)	0.99	1.0 (0.6 – 1.7)	1.0
		dabrafenib + trametinib	21 (21)	7.3	0.7 (0.4 – 1.1)	0.08	0.8 (0.5 – 1.3)	0.33
WHO performance status†	146	0-1	118 (28)	5.5	1		1	
		2-3	81 (19)	3.6	1.5 (0.9 – 2.3)	0.11	1.6 (0.9 – 2.9)	0.09
Lactate dehydrogenase	138	≤ ULN	59 (79)	6.0	1		1	
		> ULN	43 (57)	3.6	1.6 (1.1 – 2.4)	**0.01**	1.2 (0.8 – 1.8)	0.45
S100B	137	≤ ULN	26 (111)	11.5	1		1	
		> ULN	19 (81)	3.8	2.6 (1.6 – 4.3)	**< 0.01**	2.3 (1.3 – 4.3)	**< 0.01**
Brain metastases ≥ 2 cm	146	No	100 (46)	5.7	1		1	
		Yes	69 (31)	3.7	1.2 (0.8 – 1.7)	0.39	1.3 (0.8 – 2.0)	0.32
Number of brain metastases	146	≤ 5	90 (56)	5.0	1		1	
		> 5	62 (38)	4.4	1.5 (1.0 – 2.2)	**0.04**	1.5 (1.0 – 2.3)	0.08
Symptoms of brain metastases	146	Asymptomatic	83 (63)	5.0	1		1	
		Symptomatic	57 (43)	4.3	1.1 (0.8 – 1.6)	0.58	0.8 (0.5 – 1.3)	0.35
Radiotherapy during TT	146	No	102 (44)	4.3	1.5 (1.0 – 2.3)	**0.03**	1.0 (0.6 – 1.6)	0.88
		Yes	70 (30)	7.1	1		1	
Dexamethasone during TT	146	No	52 (94)	5.8	1		1	
		Yes	36 (64)	4.4	1.1 (0.8 – 1.7)	0.50	1.1 (0.7 – 1.7)	0.66

† The World Health Organization (WHO) performance status of 0 indicates that the patient is asymptomatic and fully active, 1 that the patient is restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, 2 ambulatory and capable of all self-care but unable to carry out any work activities and 3 > 50% in bed, but not bed bound. Capable of only limited self-care, confined to bed or chair 50% or more of waking hours.
CI; confidence interval, HR; hazard ratio, OS; overall survival, TT; targeted therapy, ULN; upper limit of normal

Overall survival

At time of analysis, 117 patients (80%) had died. All but two deaths were due to metastatic melanoma. Median OS of the entire cohort was 6.6 months (95% CI, 5.7 – 7.4). Median OS of patients treated with dabrafenib + trametinib was 11.2 months (95% CI, 6.8 – 15.7), 8.8 months for patients treated with dabrafenib only (95% CI, 3.9 – 13.7) and 5.7 months for patients treated with vemurafenib (95% CI, 4.6 – 6.8). A significantly longer OS was observed in the dabrafenib + trametinib group as compared to the vemurafenib group (HR for death, 0.52; 95% CI, 0.30 – 0.89; $p = 0.02$). No significant difference was seen between dabrafenib + trametinib and dabrafenib only (HR for death 0.54; 95% CI 0.26 – 1.1; $p = 0.10$) (Figure 1). Moreover, no significant difference was found between the median OS of sBM and asymptomatic BM patients; 6.6 months (95% CI 5.6 – 7.6) and 6.4 months (95% CI 4.2 – 8.5; $p = 0.22$) respectively.

Prognostic factors associated with overall survival

A normal serum LDH level, a normal serum S100B level, ≤5 BM, RT during TT, no use of dexamethasone during TT and treatment after failing TT were significant favorable prognostic factors in univariate Cox regression analysis. Equal to or less than 5 BM, RT during TT, and no use of dexamethasone during TT and treatment after failing TT remained independent favorable prognostic factors for OS (Table 5). Patients that had 3 or 4 favorable prognostic factors had a median OS of 15.1 months (95% CI, 9.7 – 20.5), compared to 6.0 months (95% CI, 5.2 – 6.7; $p < 0.01$) for patients with 0 – 2 favorable prognostic factor(s) (Figure 2).

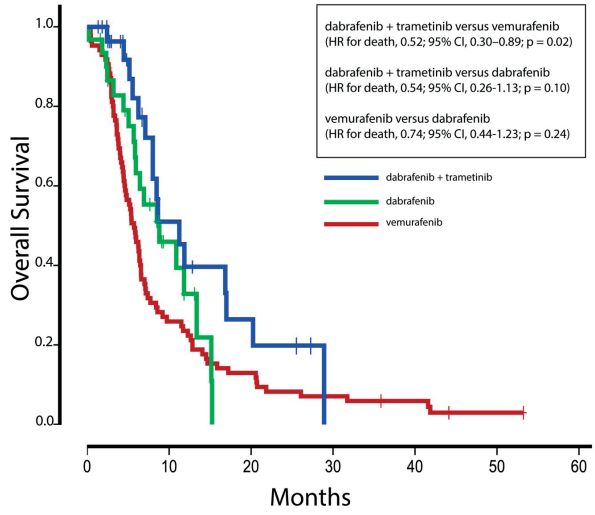

Figure 1. Kaplan-Meier overall survival curve per treatment group
Overall survival curve showing in red patients treated with vemurafenib, in green patients treated with dabrafenib and in blue patients treated with the combination of dabrafenib + trametinib.

Table 5. Univariate and multivariate Cox regression analysis for overall survival

					Univariate analysis		Multivariate analysis	
Parameter	Total *n*	Categories	*n* (%)	Median OS (months)	95% CI	*p* value	HR (95% CI)	*p* value
Treatment	146	vemurafenib	85 (31)	5.7	1		1	
		dabrafenib	30 (58)	8.8	0.8 (0.5 – 1.3)	0.27	0.8 (0.4 – 1.4)	0.39
		dabrafenib + trametinib	21 (21)	11.2	0.5 (0.3 – 0.9)	**0.02**	0.6 (0.3 – 1.1)	0.09
WHO performance status†	146	0-1	118 (28)	7.0	1		1	
		2-3	81 (19)	5.4	1.5 (1.0 – 2.5)	0.07	1.6 (0.9 – 2.9)	0.11
Serum lactate dehydrogenase	138	≤ ULN	59 (79)	7.7	1		1	
		> ULN	43 (57)	5.9	1.9 (1.3 – 2.8)	**< 0.01**	1.3 (0.8 – 2.1)	0.23
Serum S100B	137	≤ ULN	26 (111)	14.7	1		1	
		> ULN	19 (81)	5.8	2.8 (1.6 – 4.7)	**< 0.01**	1.8 (0.9 – 3.4)	0.09
Brain metastases ≥ 2 cm	146	No	100 (46)	7.0	1		1	
		Yes	69 (31)	6.2	1.2 (0.8 – 1.8)	0.30	1.1 (0.7 – 1.6)	0.82
Number of brain metastases	146	≤ 5	90 (56)	8.0	1		1	
		> 5	62 (38)	5.9	1.8 (1.2 – 2.6)	**< 0.01**	1.6 (1.0 – 2.5)	**0.04**
Symptoms of brain metastases	146	Asymptomatic	83 (63)	6.6	1		1	
		Symptomatic	57 (43)	6.4	1.3 (0.87 – 1.8)	0.22	1.0 (0.6 – 1.5)	0.92
Radiotherapy during TT	146	No	102 (44)	5.7	2.2 (1.5 – 3.4)	**< 0.01**	1.9 (1.1 – 3.1)	
		Yes	70 (30)	11.5	1		1	**0.02**
Treatment after progression of TT	146	No	108 (73)	5.8	2.2 (1.4 – 3.4)	**< 0.01**	2.4 (1.4 – 4.0)	**< 0.01**
		Yes	38 (27)	12.3	1		1	
Dexamethasone during TT	146	No	52 (94)	8.6	1		1	
		Yes	36 (64)	5.9	1.5 (1.0 – 2.2)	**0.04**	1.6 (1.0 – 2.5)	**0.04**

† The World Health Organization (WHO) performance status of 0 indicates that the patient is asymptomatic and fully active, 1: patient is restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, 2: ambulatory and capable of all self-care but unable to carry out any work activities and 3: > 50% in bed, but not bed bound. Capable of only limited self-care, confined to bed or chair 50% or more of waking hours.
CI; confidence interval, HR; hazard ratio, OS; overall survival, TT; targeted therapy, ULN; upper limit of normal

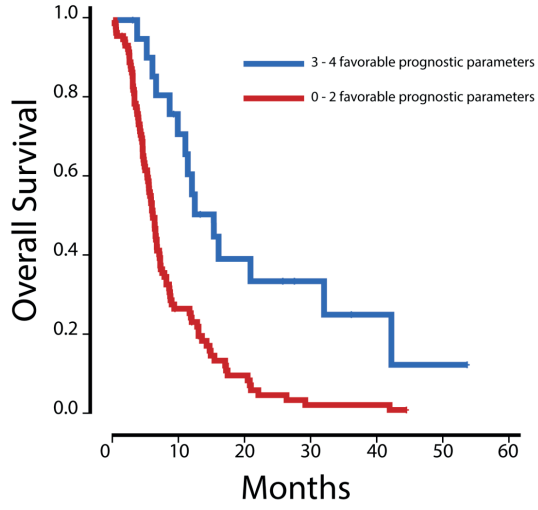

Figure 2. Kaplan-Meier overall survival curve
Kaplan-Meier overall survival curve showing in blue: patients (n = 22) with 3 or 4 favorable prognostic parameters and in red: patients (n = 124) with 0 – 2 favorable prognostic parameters. Median survival for patients with 3 or 4 favorable prognostic factors was 15.1 months (95% CI 9.7 – 20.5) and for patients with 0 – 2 prognostic factors 6.0 months (95% CI 5.2 – 6.7).
Independent favorable prognostic parameters for OS: equal to or less than 5 BM, RT during TT, no dexamethasone during TT and therapy after failing TT.

DISCUSSION

In this retrospective clinical study we analysed the effects of TT in patients with (a) symptomatic BM from BRAF-mutated malignant melanoma in three groups: vemurafenib alone, dabrafenib alone and the combination of dabrafenib + trametinib. We found a median OS of 6.6 months (95% CI 5.7 – 7.4) for all patients with a significant difference in OS between BM patients treated with dabrafenib + trametinib versus vemurafenib (HR for death, 0.52; 95% CI, 0.30 – 0.89; p = 0.02). The significantly higher OS in patients with BM from melanoma treated with dabrafenib + trametinib versus vemurafenib is an important finding. Our data are in concordance with the large COMBI-V, COMBI-D and the recently published COMBI-MB trial showing activity of dabrafenib + trametinib in BRAF-mutated melanoma patients with BM with a manageable safety profile [18-20]. In the COMBI-V and COMBI-D trials objective response rates, PFS and OS in patients with metastasized melanoma, including pre-treated stable BM, were significantly higher in the dabrafenib + trametinib group versus the vemurafenib group (COMBI-V) or the dabrafenib only group (COMBI-D) [19, 20]. The COMBI-MB trial included patients with asymptomatic BM (n = 108) and a small group with sBM (n = 17). Overall intracranial response (CR + PR) in the asymptomatic BRAF V600E mutated BM patients was 58% and 56% in patients with, respectively without, previous local RT whereas in the sBM group it was 59%. Intracranial

response in the dabrafenib + trametinib group in our group is somewhat lower: 47% in asymptomatic BM (n = 9/19) and 50% in the sBM (n = 4/8), which may be due to the small patient numbers.

The main limitations of our study are indeed that our patient groups are both small (vemurafenib n = 85; dabrafenib n = 31 and dabrafenib + trametinib n = 30) and heterogeneous, in particular with respect to previous RT treatment and that our data are obtained in a retrospective way. However, our results are in line with the large melanoma trials that dabrafenib + trametinib is the treatment of choice in patients with BRAF-mutated (a) symptomatic melanoma BM. Symptoms due to BM were not an unfavourable prognostic factor for intracranial and extracranial PFS and OS, although the use of dexamethasone was (only for OS). Forty-six percent of all sBM patients showed improvement of neurological symptoms and 45% of sBM patients that were on dexamethasone could stop this after start of TT, which means that TT is an effective palliative treatment. No significant impact of RT during TT was seen on the improvement of neurological symptoms but only 30% of patients received RT during TT in our study. Narayana et al. (2013) showed an improvement of neurological symptoms in 64% of patients with BM from melanoma treated with vemurafenib and radiation, but the contribution of TT and radiotherapy in their study is unknown [21].

Cox regression analysis demonstrated that a normal serum S100B level was an independent favourable prognostic factor both for intracranial PFS and extracranial PFS but not for OS. For OS ≤5 BM, RT during TT, no dexamethasone use during TT and (immune)therapy after tumor progression on TT were independent favourable prognostic factors. Median survival was 15.1 months in patients with 3 or 4 favourable prognostic factors and 6.0 months in patients with 0 – 2 favourable prognostic factors. Recent data showed that normal baseline serum LDH and metastases at < 3 organ sites are factors predictive for durable outcome (≥ 3 years) in patients with metastasized melanoma treated with TT [20]. Overall survival of patients with melanoma BM seems merely dependent on BM characteristics (number of BM, treatment for BM during TT (RT, no dexamethasone)) and immunotherapeutic treatment after PD and less on serum S100B level and LDH levels or type of TT treatment, the latter being only significant in univariate Cox regression analysis. Again, our results should be interpreted with caution because of the relatively low patient numbers. Therefore, it will be important to confirm the relevance of the above-mentioned prognostic factors in larger patient studies.

CONCLUSION

In conclusion, our data support that dabrafenib + trametinib is the treatment of choice in patients with both asymptomatic and symptomatic BRAF-mutated melanoma BM. Favourable prognostic factors for OS were ≤ 5 BM, RT during TT, no dexamethasone during TT and subsequent (immuno)therapy after failing TT. Patients with sBM show high clinical neurological benefit of TT, with almost 50% showing an improvement of neurological symptoms.

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Chapter 5

Targeted treatment and immunotherapy in leptomeningeal metastases from melanoma

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ABSTRACT

Background

Historically leptomeningeal metastases (LM) from melanoma has a poor prognosis, with a median survival of only two months despite treatment. Targeted therapy and immune checkpoint inhibitors are promising new treatment options in advanced melanoma. We sought to determine the impact of targeted therapy and immunotherapy on the outcome of melanoma patients with LM, and to evaluate the influence of prognostic factors.

Patients and Methods

We analyzed a series of 39 consecutive patients diagnosed with LM from melanoma between May 2010 and March 2015 treated at the Netherlands Cancer Institute. Thirty-four of these patients also had brain metastases. Statistical analyses assessed the influence of clinical and biological characteristics on survival.

Results

Median overall survival of the entire cohort was 6.9 weeks (95% CI 0.9 – 12.8). Due to a poor performance status or rapidly progressive disease, fourteen patients received no treatment. Median overall survival of untreated patients after the diagnosis of LM was 2.9 weeks versus 16.9 weeks for treated patients ($p < 0.001$). Median survival of 21 patients treated with systemic targeted therapy and/or immunotherapy, with or without RT was 21.7 weeks (range 2 – 235 weeks). Five patients had LM without brain metastases. Three of these patients died within three weeks before any treatment was given, whereas two patients are in ongoing remission for 26 weeks (following dabrafenib) and 235 weeks (following WBRT and ipilimumab). Elevated serum LDH and S100B at diagnosis of LM were associated with shorter survival.

Conclusion

Leptomeningeal metastases from melanoma still has an extremely poor prognosis. As observed in extracranial metastatic disease, new treatment modalities such as systemic targeted therapy and immune checkpoint inhibitors seem to increase overall survival in LM, and may result in long-term remission. These new treatment options should be considered in patients with LM.

INTRODUCTION

Leptomeningeal metastases (LM) is one of the most devastating complications in solid tumors. It is clinically detected in about 5% of patients with cancer, mainly in breast cancer, lung cancer and melanoma [1]. Higher numbers are reported in autopsy series of patients with brain metastases [2, 3]. Difficulties to differentiate symptoms of LM from those caused by brain metastases (BM) may contribute to this underestimation, but limited sensitivity of diagnostic tests may also play a role. Besides, specific clinical signs are absent in at least 25% of patients at the diagnosis of LM [4]. The golden standard for the diagnosis of LM is demonstration of tumor cells in the cerebrospinal fluid (CSF). Sensitivity of CSF cytology is 50% on first lumbar puncture, and increases to 80% after repeated punctures [5]. The diagnosis can also be made by magnetic resonance imaging (MRI). MRI has a sensitivity and specificity of about 75% [6]. On clinical suspicion of LM, typical leptomeningeal contrast enhancement on MRI is considered diagnostic. Median survival of untreated patients with LM from solid tumors is only 4 to 6 weeks, usually due to progressive neurologic dysfunction [7]. Focal radiotherapy (RT) can relieve neurologic symptoms, but has no significant effect on survival [8]. Intrathecal chemotherapy (IT) is considered the mainstay of treatment of LM but its efficacy remains uncertain [5]. In LM from breast cancer, systemic treatment appeared at least as effective but less toxic than IT chemotherapy, suggesting that the blood-CSF barrier is not the crucial factor in LM [9]. Only a few series of patients with LM from melanoma have been published with reported median overall survival of 8 to 10 weeks [10, 11].

Two new treatment modalities have significantly improved survival in patients with advanced melanoma. Vemurafenib and dabrafenib, inhibitors of the mutated BRAF protein (evident in 50% of melanoma patients) have shown impressive albeit temporary responses, also in BM [12, 13]. The second new treatment strategy is the application of immune checkpoint inhibitors, like ipilimumab and nivolumab that enhance the anti-tumor T-cell response and, importantly, induce long lasting responses in a subset of patients. A complete response in a patient with LM from melanoma treated with radiotherapy and ipilimumab was reported earlier [14]. In this study we sought to determine the influence of new treatment modalities and of prognostic factors on outcome in patients with LM.

MATERIAL AND METHODS

A cohort of 39 consecutive patients diagnosed with LM from melanoma at the Netherlands Cancer Institute between May 2010 and March 2015 was analyzed. Diagnosis was based on MRI and/or CSF cytology.

Data collected included age, gender, date of diagnosis of melanoma, date of diagnosis of LM, performance status at diagnosis of LM, presence of brain metastases, number (1, 2-5 or > 5) and volume (< or > 2 cm diameter) of brain metastases, neurological signs and symptoms at diagnosis of LM, use of corticosteroids, CSF results (leukocyte count, protein, glucose, LDH), treatment for brain metastases and/or LM, date of death or last follow-up, serum blood lactate dehydrogenase (LDH) and S100B levels at diagnosis of LM.

Statistical analysis

Survival was measured from the date of diagnosis of LM to death, or last follow-up. Kaplan-Meier curves were made to estimate survival percentages. A *p*-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 22 (IBM corp., Armonk, NY, USA). Log-rank test was used to assess the influence of baseline characteristics on survival.

RESULTS

Patient characteristics

Patient characteristics at time of diagnosis of LM are summarized in Table 1. Median time from diagnosis of melanoma to LM was 3.2 years (range 0 – 29). At time of data analysis (June 2015) four patients were still alive. At diagnosis of LM ten patients (26%) had a WHO performance status (PS) of 2 (26 %) and six patients (15%) a PS of 3. The diagnosis LM was established in 36 patients (92%) by MRI and in three patients (8%) by CSF cytology. Thirty-three patients (85%) had neurological symptoms. The most common LM symptoms at diagnosis were headache (46%), nausea and vomiting (44%), gait difficulty (39%) and seizures (31%). In six asymptomatic patients, diagnosis of LM was an incidental finding at screening or follow-up MRI. Thirty-four patients (87%) also had brain metastases (BM). Ten patients (29%) were not treated for their BM. Thus, twenty-four patients (71%) were treated for BM; 16 patients received RT and 21 patients systemic therapy.

Table 1. Patient characteristics and univariate analysis of factors associated with survival

	No. of patients (%)	Median OS (95% CI)	p value
Age, years	**52.9 years (range, 26-84)**		
Sex			
Male	**23 (59)**	**6.4 (1.5 – 11.3)**	**0.8**
Female	**16 (41)**	**8.0 (0 – 17.5)**	
WHO performance status			
0-1	**22 (56)**	**18.6 (9.8 – 27.9)**	**< 0.001**
2-3	**16 (41)**	**3.6 (2.7 – 4.4)**	
Unknown	**1 (3)**		
Lactate dehydrogenase			
0-248 U/L (normal)	**19 (49)**	**18.6 (10.8 – 26.9)**	**< 0.001**
> 248 U/L (elevated)	**14 (36)**	**3.1 (1.6 – 4.7)**	
Unknown	**6 (15)**		
S100B			
0-0.10 µg/L (normal)	**9 (23)**	**24.9 (15.7 – 34.0)**	**0.03**
> 0.10 µg/L (elevated)	**23 (59)**	**5.1 (1.8 – 8.5)**	
Unknown	**7 (18)**		
Brain metastases			
Yes	**34 (87)**	**6.9 (1.1 – 12.6)**	**0.43**
No	**5 (13)**	**3.1 (1.3 – 5.0)**	
Number of brain metastases*			
None	**5 (13)**	**HR 0.5 (0.1 – 1.7)**	**0.24**
1	**2 (5)**	**HR 1.6 (0.4 – 6.8)**	**0.54**
2-5	**9 (23)**	**HR 0.5 (0.2 – 1.1)**	**0.09**
> 5	**23 (59)**	**1 (ref)**	
Treatment for LM			
Yes	**25 (64)**	**16.8 (11.6 – 22.1)**	**< 0.001**
No	**14 (36)**	**2.9 (0 – 6.0)**	
Treatment for LM*			
No treatment	**14 (36)**	**1 (ref)**	
RT	**4 (10)**	**HR 0.53 (0.2 – 1.7)**	**0.28**
Systemic	**10 (26)**	**HR 0.17 (0.06 – 0.5)**	**0.001**
RT + systemic	**11 (28)**	**HR 0.07 (0.02 – 0.2)**	**< 0.001**
Symptoms of LM			
Yes	**33 (86)**	**6.4 (2.6 – 10.3)**	**0.45**
No	**6 (14)**	**11.0 (0 – 40.0)**	

Abbreviations: HR, Hazard Ratio; LM, leptomeningeal metastases; OS, Overall Survival; RT, radiotherapy; WHO, World Health Organization

* Hazard Ratio

Treatment and survival

Twenty-five patients (64%) were treated for LM (for characteristics of treated patients see Table 2).

Table 2. Characteristics of the treated patients at time of diagnosis of LM

Patient no.	PS at diagnosis	Age at diagnosis (years)	Symptoms of LM	Treatment	Time from LM to death (weeks)
1	0	50	Cerebral	vemurafenib, WBRT	21.7
2	1	66	Cerebral	WBRT, ipilimumab	235.1+
3	2	61	Cerebral	dabrafenib+trametinib	3.1
4	1	39	Cerebral and cranial nerves	ipilimumab, WBRT	15.1
5	1	44	Cerebral	vemurafenib, WBRT	15.3
6	0	59	Cerebral	dabrafenib+trametinib, WBRT	24.9
7	1	64	None	vemurafenib, ipilimumab	26.0
8	0	64	Cerebral	WBRT, vemurafenib	18.9
9	1	47	Cerebral	WBRT	2.3
10	0	65	Cerebral and cranial nerves	ipilimumab	6.0
11	0	48	None	vemurafenib	48.4
12	1	49	Cerebral	ipilimumab, WBRT	10.0
13	0	50	None	WBRT, DTIC, ipilimumab	68.6
14	3	51	Cerebral	WBRT	3.6
15	0	50	Cerebral and cranial nerves	WBRT, dabrafenib+trametinib, ipilimumab	47.0
16	0	52	Cerebral	vemurafenib, WBRT	33.6
17	0	49	Spinal	spinal RT, dabrafenib+trametinib	61.9+
18	3	67	Cerebral	WBRT	15.9
19	2	26	Cerebral and cranial nerves	vemurafenib	3.9
20	2	49	Cerebral and cranial nerves	SRT	5.1
21	3	73	Cerebral	vemurafenib	16.9
22	1	57	Cerebral	ipilimumab	6.4
23	1	60	Cerebral and cranial nerves	ipilimumab	2.0
24	1	52	Cerebral, cranial nerves and spinal	dabrafenib, ipilimumab	16.4+
25	0	77	Spinal	dabrafenib	26.4+

+: patient alive at time of analysis

Abbreviations: LM, leptomeningeal metastases; PS, performance status; SRT, stereotactic radiotherapy; RT, radiotherapy; WBRT, whole brain radiotherapy

Treatment for LM included cranial or spinal RT in 15 patients and systemic therapy in 21 patients. No IT chemotherapy was given. Of the 21 systemically treated patients, eight patients were treated with a BRAF inhibitor (vemurafenib or dabrafenib), three patients were treated with a BRAF inhibitor in combination with a MEK inhibitor (dabrafenib and trametinib), six received ipilimumab (a CTLA-4 monoclonal antibody), two patients were treated with ipilimumab followed by a BRAF inhibitor, one patient was treated with dabrafenib in combination with trametinib followed by ipilimumab, and one patient was treated with dacarbazine followed by ipilimumab. Thus, a BRAF inhibitor was given in 14 patients, and ipilimumab in 10 patients. Fourteen patients (36%) did not receive any therapy after the diagnosis of LM due to rapid disease progression or poor performance. Of the 16 patients with a PS of 2 or 3, only six (38%) received treatment for LM (three RT and three systemic treatment). Patients with a performance status of 2 or 3 had a significantly worse median overall survival compared to patients with a performance status of 0 or 1 (3.6 versus 18.8 weeks $p < 0.001$). There was no significant difference in median survival between untreated patients with a PS of 2 or 3 and the six patients who received treatment (1.9 versus 3.9 weeks $p = 0.075$). Median overall survival for all patients was 6.9 weeks (95% CI 0.9 – 12.8) (Figure 1).

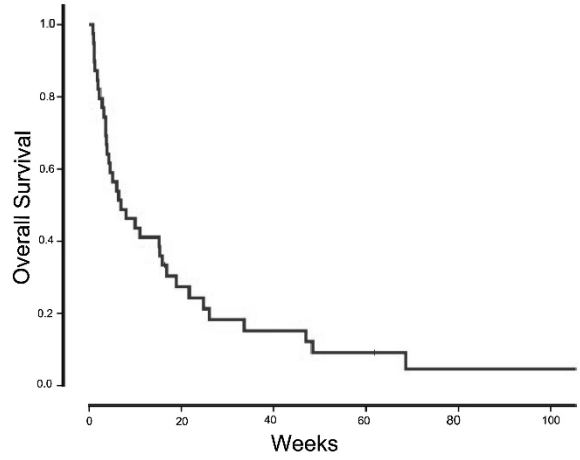

Figure 1. Kaplan-Meier curve for overall survival in weeks. Median overall survival = 6.9 weeks

There was no significant difference in survival in patients with or without neurological symptoms ($p = 0.45$). There was also no difference in survival in patients with or without corticosteroids ($p = 0.85$). Volume of BM was not significantly related to overall survival ($p = 0.54$). Of the fourteen patients who did not receive any therapy for their LM, median survival was 2.9 weeks (95% CI 0 – 6.0) versus 16.9 weeks for treated patients (95% CI 11.6 – 22.1) ($p < 0.001$). Median survival of the 21 patients treated with a BRAF inhibitor and/or ipilimumab was 21.7 weeks (range 2 – 235 weeks). Median survival of the 14 patients

in which treatment included a BRAF inhibitor (with or without a MEK inhibitor) was 24.9 weeks (range 3 – 62 weeks) (with RT 25 weeks, without RT 16 weeks). Median survival of the ten patients in which treatment included ipilimumab was 15.8 weeks (range 2 – 235 weeks) (with RT 47 weeks, without RT 6 weeks). Median survival of the four patients treated with RT only was 4.3 weeks (range 2 – 16 weeks).

Serum lactate dehydrogenase (LDH) at diagnosis of LM was available from 33 patients (85%); fourteen of these (42%) had an increased LDH (> 248 U/L). Patients with LM and an increased LDH had a significant shorter survival of 3.1 weeks (95% CI 1.5 – 4.7) compared to 18.9 weeks for patients with normal LDH (95% CI 10.8 – 26.9, $p < 0.001$, Figure 2).

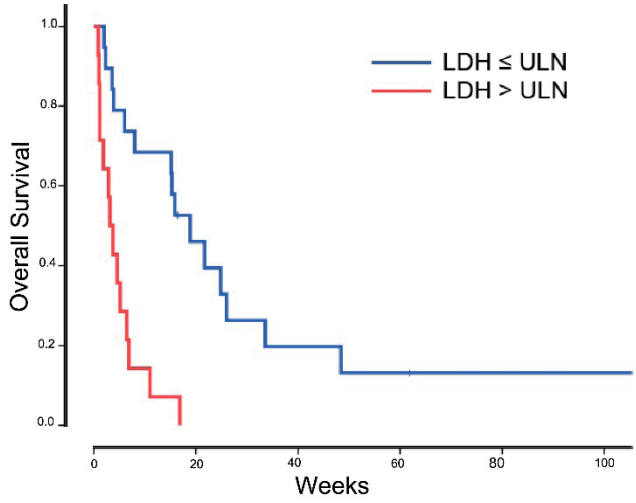

Figure 2. Kaplan-Meier curve for LDH. Median overall survival in patient group with LDH ≤ ULN = 18.9 weeks ($n = 19$), median overall survival in patient group with LDH > ULN = 3.1 weeks ($n = 14$). LDH, lactate dehydrogenase; ULN, upper limit of normal.

Patients with increased LDH were less likely to receive any treatment modality for LM; four of 14 patients with increased LDH were treated versus 18 of 19 patients with a normal LDH ($p < 0.001$). Serum S100B values were available from 32 (82%) patients at time of LM diagnosis. Nine patients (28%) had a normal serum S100B level, and 23 (72%) had an increased serum S100B level. Patients with a normal serum S100B level had a median overall survival of 24.9 weeks (95% CI 15.7 – 34.0) versus 5.1 weeks (95% CI 1.7- 8.5) for patients with an increased S100B level ($p = 0.04$). Thirty-five patients had died at time of analysis. Twenty-four patients (68%) died primarily of neurological progression, eight patients (23%) of both intracranial and extracranial progression, while three deaths (9%) were not directly tumor related. Of the twenty-four patients who primarily died of neurological progression, two patients died of progression of brain metastases, 11 patients due

to progression of LM while in 11 patients cause of death could not be attributed to LM or BM with certainty. Of the four patients still alive at time of analysis one patient was treated with local RT at L2-S5 (1x8 Gy) followed by dabrafenib and trametinib for widespread spinal LM causing a cauda equina syndrome (Figure 3). An ongoing response of 62 weeks was achieved of LM and of asymptomatic brain metastases.

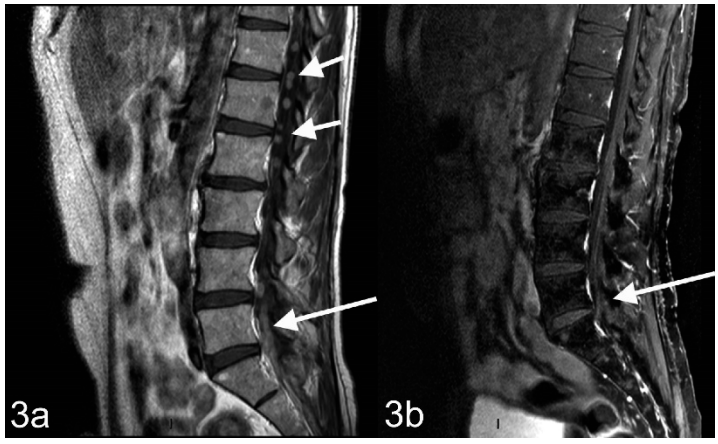

Figure 3. Post-gadolinium sagittal MRI T1-weighted images of Th11-S2, demonstrating thickening and enhancement of the cauda equine nerve roots (long arrows) and enhancing intradural nodules (short arrows) in December 2013 before RT L2-S5 and start of dabrafenib and trametinib (a), and only slight enhancement of lumbosacral nerve roots in January 2015 during treatment with dabrafenib and trametinib (Post-gadolinium sagittal MRI T1 with fat-suppression) (b).

Patient characteristics and treatment characteristics in patients with LM only

Five patients had LM without brain metastases. Three of these five patients presented with headache, vomiting, seizures and cranial nerve involvement with rapid clinical deterioration, and died within three weeks before any specific treatment was given.

The fourth patient presented with weight loss, fatigue and pain in both legs twenty-eight years after resection of a melanoma on his back. A PET-CT scan showed metastases in lymph nodes, kidneys, peritoneum, small bowel, and subcutaneously and pathologic FDG activity in the lumbar spinal canal. Additional MRI of the lumbar spine showed diffuse LM. He is currently being treated with dabrafenib, resulting in a neurological and radiological partial response for six months now. He did not receive local RT.

The fifth patient presented with progressive nausea and vomiting. Cerebral MRI showed multifocal enhancement of the leptomeninges consistent with the diagnosis of LM. She also had lymph node and lung metastases. She was treated with WBRT and 4 cycles of

ipilimumab, resulting in a complete radiological and clinical remission (see also [14]). She is free of disease for four and a half years now.

DISCUSSION

This retrospective study confirms the well-known dismal outcome of LM, and shows that for patients with melanoma, outcome is even worse compared to patients with LM from other solid tumors. More than one third of our patients had a performance status too poor for anti-tumor treatment and died in a median time of less than three weeks. The typical steep decline in the survival curve for about one third of the patients is consistent with data from literature [5, 8, 11]. A remarkable and encouraging new finding in our study are the long-term survivors when patients are being treated with targeted treatment or immunotherapy. Moreover, the median survival of 22 weeks following these new therapies compares favorably to reported results of IT chemotherapy for LM from melanoma [10, 11]. Earlier studies on immunotherapy for LM from melanoma included IT interleukin-2 (IL-2), that showed incidental responses, but also marked toxicity [11, 15]. The new checkpoint inhibitor ipilimumab has shown impressive responses in patients with advanced melanoma with a four months increase in median survival and, importantly about 20% long term survival [16]. Ipilimumab enhances anti-tumor T cell activation in the lymph nodes. As activated T-cells can cross the blood-brain barrier or blood-CSF barrier, these barriers seem less relevant for a response within the CNS. In patients with BM not requiring steroids, the intracranial response after ipilimumab approximated the extracranial response (RR 24% vs 27%) [17]. Combination with RT may increase the response by the so-called abscopal effect, i.e. increased release of tumor antigen by RT can increase antigen presentation to T cells [18]. Responses to immune checkpoint inhibitors can be delayed as first an increase of activated T cells at the tumor location is needed. In contrast, the response of metastasized melanoma to BRAF inhibitors is prompt. The response rate is about 50% in advanced BRAF mutated melanoma [19]. Although vemurafenib does not cross an intact blood-brain barrier, vemurafenib has shown to be effective in brain metastases from melanoma, but also high rates of intracranial relapse during extracranial disease control were observed [20]. Dabrafenib also does not cross an intact blood-brain barrier but similar intracranial and extracranial responses (+/- 40%) were reported after first-line treatment with dabrafenib [12, 21]. A response of LM to BRAF inhibitors as single agent has not been reported yet. In the present study an ongoing response of 62 weeks of LM outside the RT portal was documented following dabrafenib and trametinib treatment, again demonstrating that the blood-CSF barrier does not exclude successful systemic treatment of overt CNS metastases. Upregulation of the MEK pathway causes

BRAF inhibitor resistance, so combination with the MEK inhibitor trametinib probably prolonged the duration of response in our patient.

At univariate analysis, elevated serum LDH and S100B levels, both markers for tumor burden in melanoma, were associated with shorter survival. Most of the patients with elevated LDH were not treated after the diagnosis LM because of poor performance status and rapid clinical deterioration. Other possible prognostic factors, like presence and kind of neurologic symptoms, use of corticosteroids, and presence, volume and number of brain metastases were not associated with survival.

CONCLUSION

Leptomeningeal metastases from melanoma still has an extremely poor prognosis. As observed in extracranial metastatic disease new treatment modalities, such as systemic targeted therapy and immunotherapy seem to increase median survival with a few months, and may result in long-term remissions. Combining these therapies with radiotherapy might enhance their efficacy. Especially in LM patients with a good performance score and low serum LDH and S100B levels these treatment options should be considered.

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Chapter 6

Vemurafenib for *BRAF* V600 mutated advanced melanoma: results of treatment beyond progression

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ABSTRACT

Background

Selective BRAF inhibition (BRAFi) by vemurafenib or dabrafenib has become approved standard treatment in *BRAF* V600 mutated advanced stage melanoma. While the response rate is high, the response duration is limited with a progression-free survival (PFS) of 5-6 months. Our observation of accelerated disease progression within some patients after stopping vemurafenib treatment has fostered the idea of treatment beyond progression (BRAFi TBP).

Method

In this retrospective study, we analyzed 70 metastatic melanoma patients, treated at our institute, who experienced progression after prior objective response upon treatment with vemurafenib. Thirty-five patients that continued treatment beyond progression are compared with 35 patients who stopped BRAFi treatment at disease progression.

Results

Median overall survival beyond documented progression was found to be 5.2 months versus 1.4 months (95% CI: 3.8-7.4 vs. 0.6-3.4; Log-Rank p = 0.002) in favour of BRAFi TBP. In the multivariate survival analysis, stopping treatment at disease progression was significantly associated with shorter survival (Hazard Ratio: 1.92; 95% CI: 1.04-3.55; p = 0.04).

Conclusion

Our results suggest that continuing vemurafenib treatment beyond progression may be beneficial in advanced melanoma patients, who prior to progression responded to vemurafenib.

INTRODUCTION

Melanoma has a rising incidence in Europe resulting in an estimated 100,300 new diagnoses and yearly 22,200 patients succumb to this disease in 2012 [1, 2]. The progress that has been made in the understanding of melanoma pathogenesis in the past decade has resulted in the development of novel targeted therapies such as vemurafenib and dabrafenib [3-5]. Both drugs inhibit the activity of mutated BRAF proteins, which are observed in 40-60% of cutaneous melanoma [6-9]. Although these selective BRAF inhibitors showed improvement in progression-free survival (PFS) and overall survival (OS), more than 50% of patients will have progressed after five to six months of treatment, highlighting the problem of acquired therapy resistance [10]. A novel combination therapy, consisting of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib, has been investigated and preliminary results point towards improvement in PFS [11], suggesting that resistance can be postponed by combining two inhibitors of the MAPK pathway.

We clinically observed at our institute that stopping BRAF inhibition (BRAFi) due to disease progression resulted often in an accelerated growth of metastases, and consecutive rapid deterioration and death of the patients. This has raised the question whether continuation of vemurafenib despite disease progression or so-called treatment beyond progression (TBP), could improve overall survival of these patients. Recent data from dabrafenib indicated that this might be indeed the case [12]. The exact mechanism behind accelerated growth of metastases after discontinuing vemurafenib is thus far unknown. One possible explanation may lie in inter-tumoral and intra-tumoral heterogeneity with tumor growth of vemurafenib resistant tumor cells, while other portions of the tumor or other metastases may still be responsive. Stopping vemurafenib based on progressive disease as a result of growth of resistant metastases may lead to sometimes rapid growth of all lesions. A study by Carlino et al. reported a marked increase in the rate of disease progression after withdrawal of MAPK inhibitors (either dabrafenib or the combination of dabrafenib plus trametinib) in patients with *BRAF*-mutant metastatic melanoma treated beyond progression [13]. The same study also showed a slower rate of disease proliferation in resistant melanoma cell lines when continuously exposed to MAPK inhibition. Another, relatively small study with 48 patients with metastatic melanoma showed a potential benefit in treatment beyond progression in patients who showed progression of disease in limited sites only, which was accessible to local therapy [14]. The possible advantages of continuing treatment with vemurafenib have not yet been extensively investigated in melanoma patients, however, results obtained from several studies focusing on other malignancies and other treatments point towards an advantage of TBP [13-21].

Here, we present our retrospective single institution analysis of vemurafenib treatment beyond progression in advanced stage *BRAF* V600 mutated melanoma patients and show a potential beneficial effect of continuation of treatment despite disease progression.

MATERIALS AND METHODS

Patients included in the analysis

This study was undertaken at the Netherlands Cancer Institute – Antoni van Leeuwenhoek. The study included 152 patients with *BRAF*-mutant metastatic melanoma, who are/were treated at our institute with vemurafenib (within the Global Safety Study, 86 patients and on prescription after approval of vemurafenib, 66 patients) between June 2010 and February 2013 [22].

Methods

Vemurafenib was given orally at a standard dose of 960 mg twice daily, unless patients experienced toxicities for which dose modification was needed. In one patient with good tolerability, vemurafenib was escalated to a dose of 1200 mg twice daily upon progression of disease. Initially, patients treated in the Global Safety Study were not permitted to continue BRAFi treatment once progression of disease set in. As of the European approval of vemurafenib in 2012, clinicians treating patients in the Global Safety Study have been permitted to continue TBP upon request to and approval by the study monitor. The rationale behind choosing which patients received TBP and which patients would not receive TBP was determined based on multiple factors including: ECOG performance status, nature of disease progression and possibility of other therapies beyond progression. TBP was defined as receiving BRAFi despite progression of disease as measured by RECIST 1.1. During therapy, patients visited the outpatient clinic every four weeks for physical examination and blood sampling. Tumor responses were assessed every eight weeks by CT-scan and in case of brain metastases also by MRI. Nature of disease progression was noted as followed: intracranial versus extracranial, nonvisceral (subcutaneous, bone and lymph node) versus visceral (lung, liver and pancreas), new and/or existing metastases and whether progression of disease was more isolated or generalized. Isolated disease progression was defined as progression with a new or an existing lesion within one site or organ, while the rest of the disease showing at least stable disease. Patient characteristics were obtained from the electronic patient records within our institute.

Statistical analysis

The primary endpoint of our retrospective analyses is OS. We performed two types of OS analyses: traditional OS (OS from start of treatment) and post-progression OS (ppOS)

defined as OS after disease progression according to RECIST 1.1. PFS was measured from the date of vemurafenib commencement until disease progression according to RECIST 1.1. Patients alive at data cut-off are marked as censored in the Kaplan-Meier survival curve. Univariate and multivariate Cox regression methods were used to estimate the hazard ratio (HR) of continuing vemurafenib beyond progression for ppOS. The following known prognostic factors were included in the multivariate analysis: age, serum LDH level, ECOG performance status, M-stage and presence of brain metastases [23-26]. A sensitivity analysis was performed where BRAFi TBP was defined as treatment beyond 0, 7, 14, 21 and 28 days of documented progression. As this was a retrospective case-control study with overall survival as primary endpoint, patients who received subsequent systemic treatment were not censored at the time of starting subsequent treatment.

RESULTS

Patient characteristics within the cohorts

In total 152 patients with *BRAF*-mutant metastatic melanoma were identified. Patients were excluded from this analysis due to absence of measurable disease according to RECIST 1.1, absence of any initial response, or due to ongoing response at data cut-off (see Figure 1). Two patients continued therapy at other institutions and were lost to follow up. From the remaining 70 patients 35 continued vemurafenib treatment (BRAFi TBP) despite disease progression and 35 discontinued vemurafenib at time of progression of disease (no

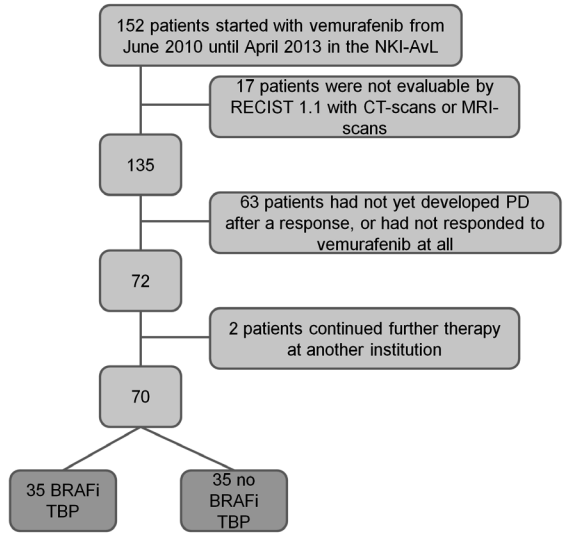


Figure 1. Flow chart showing process of patient selection

BRAFi TBP), thereby serving as a control group. In the BRAFi TBP group 24 patients were in the Global Safety Study and 11 patients received vemurafenib after approval by EMA. For the patients that stopped vemurafenib at disease progression 26 were in the Global Safety Study and 9 received vemurafenib after approval by EMA. Patient characteristics at time of study commencement are shown in Table 1.

The median follow-up was 22 months at data cut-off as of February 2014. Twenty-nine patients in the BRAFi TBP group had died, while this was the case for 34 patients in the control group. The patients' characteristics of both cohorts at the time of disease progression are summarized in Table 2. Fifty-nine percent of the patients were men and the mean age of the entire cohort was 55 years. As shown in Table 2, significant imbalances were found concerning the distribution of ECOG performance status ($p < 0.001$), M-stage ($p = 0.011$) and serum lactate dehydrogenase (LDH) level ($p = 0.037$) between the two groups. The presence of brain metastases was similar in both cohorts. In both groups subsequent therapies were started at discretion of the treating physician, which was slightly more frequent (not significant, $p = 0.46$) in the no TBP group (Table 3).

Table 1. Patient characteristics at study commencement

	BRAFi TBP (n = 35) at baseline	**No BRAFi TBP (n = 35) at baseline**	**p value**
Age mean (SD)	**51.5 (13)**	**57.1 (13)**	**0.077**
Gender			**0.628**
Male	**19 (54)**	**22 (63)**	
Female	**16 (46)**	**13 (37)**	
ECOG performance status			**0.106**
0-1	**34 (97)**	**29 (83)**	
2-3	**1 (3)**	**6 (17)**	
Lactate dehydrogenase			**0.216**
0-250 U/L	**20 (57)**	**15 (42)**	
251-500 U/L	**11 (31)**	**10 (29)**	
> 500 U/L	**4 (11)**	**10 (29)**	
Unknown	**0 (0)**	**0 (0)**	
M-stage			**0.152**
M1a	**3 (9)**	**0 (0)**	
M1b	**5 (14)**	**3 (9)**	
M1c	**27 (77)**	**32 (91)**	
Brain metastases			**0.808**
Yes	**22 (63)**	**20 (57)**	
No	**13 (37)**	**15 (43)**	

Abbreviations: BRAFi, BRAF inhibitor; ECOG PS, Eastern Cooperative Oncology Group performance status; TBP, treatment beyond progression.

Table 2. Patient characteristics at the time of BRAF inhibitor disease progression

	BRAFi TBP (n = 35) at progression	No BRAFi TBP (n = 35) at progression	p value
Age mean (SD)	52.5 (13)	58.2 (13)	0.073
Gender			0.628
Male	19 (54)	22 (63)	
Female	16 (46)	13 (37)	
ECOG performance status			< 0.001
0-1	34 (97)	21 (60)	
2-3	1 (3)	14 (40)	
Lactate dehydrogenase			0.037
0-250 U/L	23 (66)	16 (46)	
251-500 U/L	8 (22)	8 (22)	
> 500 U/L	2 (6)	10 (29)	
Unknown	2 (6)	1 (3)	
M-stage			0.011
M1a	3 (9)	0 (0)	
M1b	4 (11)	0 (0)	
M1c	28 (80)	35 (100)	
Brain metastases			1
Yes	17 (49)	18 (51)	
No	18 (51)	17 (49)	

Abbreviations: BRAFi, BRAF inhibitor; ECOG PS, Eastern Cooperative Oncology Group performance status; TBP, treatment beyond progression.

Nature of disease progression upon BRAFi

Data regarding the nature of disease progression are shown in Table 4. When looking at type of progression of disease 29 of 70 patients (41%) progressed in existing metastases only, while only 9 of 70 patients (13%) had progression of disease due to development of only new metastases. Most patients, 39 of 70 (56%), had generalized progression of disease (i.e. progression in more than one site or organ). There was a significant difference in the two groups: in the BRAFi TBP group 14 of 35 (40%) patients had generalized progression, while this was 25 of 35 (71%) patients in the group that stopped BRAFi treatment at progression (p = 0.015).

When looking more closely, the majority of patients, 42 of 70 (60%), progressed at extracranial sites only, while 10 of 70 patients (14%) progressed only intracranially. Eighteen of 70 patients (26 %) progressed at both intracranial and extracranial sites. Intracranially, 20 of 70 (29%) patients showed progression in existing lesions, while 24 of 70 (34%) patients progressed due to the formation of new metastases. Interestingly, when comparing the site of progression of disease between the two groups there was a significant difference in the nature of progression. In the cohort that stopped BRAFi treatment upon progression

26 of 35 patients (74%) showed progression in existing nonvisceral metastases, while this was only in 16 of 35 patients (46%) in the cohort that had BRAFi TBP (p = 0.03).

Table 3. Management after progression of disease from BRAF inhibitor

Factor	No. of Patients (%)			
	Total	BRAFi TBP	No BRAFi TBP	p value
Total	70 (100)	35 (50)	35 (50)	
Treatment after vemurafenib				0.46
Ipilimumab	20 (29)	10 (29)	10 (29)	
DTIC	3 (4)	1 (3)	2 (6)	
Temozolomide	1 (1)	0 (0)	1 (3)	
Anti PD-1	2 (3)	1 (3)	1 (3)	
Tumor Infiltrating Lymphocytes	1 (1)	0 (0)	1 (3)	
None	43 (61)	23 (65)	20 (57)	
Disease progression amenable to local				0.03
treatment	38 (54)	14 (40)	24 (69)	
No	32 (46)	21 (60)	11 (31)	
Yes				
Disease progression treated locally				0.02
No	39 (56)	14 (40)	25 (71)	
Yes	31 (44)	21 (60)	10 (29)	
Patients with intracranial disease progression				
n = 28				-
Intracranial surgery	28 (100)	13 (100)	15 (100)	
No	0 (0)	0 (0)	0 (0)	
Yes				
Intracranial SRS				0.21
No	26 (93)	11 (85)	15 (100)	
Yes	2 (7)	2 (15)	0 (0)	
Intracranial WBRT				0.11
No	18 (64)	6 (46)	12 (80)	
Yes	10 (36)	7 (54)	3 (20)	
Patients with extracranial disease progression				
n = 60				0.05
Extracranial surgery	52 (87)	23 (77)	29 (97)	
No	8 (13)	7 (23)	1 (3)	
Yes				
Extracranial XRT				0.75
No	48 (80)	25 (83)	23 (77)	
Yes	12 (20)	5 (17)	7 (23)	

Abbreviations: BRAFi, BRAF inhibitor; SRS; stereotactic radiosurgery; TBP, treatment beyond progression; WBRT, whole-brain radiotherapy; XRT, radio-therapy.

Local treatment after BRAFi progression of disease

Twenty-eight patients had intracranial disease progression. Of those 28 patients 12 (43%) received local treatment to progressing sites. Two patients (7%) received stereotactic radiotherapy and 10 patients (36%) received whole-brain radiotherapy. No patient had

intracranial surgery for intracranial disease progression. Of 60 patients who had extracranial disease progression, 20 (33%) received local treatment for progressing sites. Eight patients (13%) underwent local surgery and 12 patients (20%) received local radiotherapy. When comparing the BRAFi TBP group to the group who stopped BRAFi treatment upon progression there was a borderline significant difference in patients who underwent extracranial surgery (7 versus 1 patient $p = 0.05$) in favor of the TBP group.

Table 4. Nature of BRAFi progression of disease

Factor	No. of Patients (%)			
	Total	BRAFi TBP	No BRAFi TBP	p value
Intracranial/extracranial disease progression	42 (60)	22 (63)	20 (57)	0.941
Extracranial only	10 (14)	5 (14)	5 (14)	
Intracranial only	18 (26)	8 (23)	10 (29)	
Extracranial and intracranial				
Type of progression				0.016
Existing lesion	29 (41)	19 (54)	10 (29)	
New lesion	9 (13)	6 (17)	3 (9)	
New and existing lesions	32 (46)	10 (29)	22 (63)	
Isolated*	31 (44)	21 (60)	10 (29)	0.015
Generalized	39 (56)	14 (40)	25 (71)	
Site of progression				
Visceral existing				1
No	35 (50)	17 (49)	18 (51)	
Yes	35 (50)	18 (51)	17 (49)	
Visceral new+				0.133
No	56 (80)	31 (89)	25 (71)	
Yes	14 (20)	4 (11)	10 (29)	
Nonvisceral existing+				0.03
No	28 (40)	19 (54)	9 (26)	
Yes	42 (60)	16 (46)	26 (74)	
Nonvisceral new+				1
No	52 (74)	26 (74)	26 (74)	
Yes	18 (26)	9 (26)	9 (26)	
Brain existing				1
No	50 (71)	25 (71)	25 (71)	
Yes	20 (29)	10 (29)	10 (29)	
Brain new				0.45
No	46 (66)	25 (71)	21 (60)	
Yes	24 (34)	10 (29)	14 (40)	

Abbreviations: BRAFi, BRAF inhibitor; TBP, treatment beyond progression.

* Isolated progression of disease was defined as progression in a new or existing lesion within one site or organ, where the rest of disease showing at least stable disease.

+ Visceral disease included lung, liver and pancreas; nonvisceral included subcutaneous, bone and lymph node disease.

Systemic treatment after BRAFi progression of disease

As previously described, at time of progression of disease according to RECIST 1.1, 35 of 70 patients (50%) continued treatment with vemurafenib. Twenty of 70 patients (29%) did not receive any subsequent treatment and 15 (21%) received other therapies such as ipilimumab, dacarbazine, temozolomide, anti-PD1 or tumor infiltrating lymphocytes. Of the 35 patients who continued BRAFi treatment despite progression of disease 12 (34%) eventually received other systemic treatment when progression was not manageable anymore with vemurafenib. Subsequent treatment included ipilimumab, dacarbazine and anti PD-1. There was no significant difference between the two groups regarding subsequent systemic treatment. The median duration of continued BRAFi TBP was 103 days (range 13-401). The median number of cycles (4 weeks vemurafenib) given in the BRAFi TBP group was 4 (range 1-14).

Clinical outcomes

Median PFS for all 70 patients was 5.2 months (Figure 2A) and thus comparable to the data observed in the phase 3 study and the global safety study [22, 24]. Median PFS within the BRAFi TBP group was significantly longer than that of patients in the no BRAFi TBP group (5.6 months vs. 4.0 months, CI: 4.4-7.5, 3.7-5.5; Log-Rank p = 0.02) (Figure 2B). This may have been the result of the difference in ECOG PS and serum LDH levels between the two groups at the start of the vemurafenib treatment. Results from the global safety study point towards a shorter PFS for these subgroups [22]. This translated also into a significantly longer median OS (Figure 2C), namely 12.8 months in the TBP group versus 6.3 months in the control group (Log-Rank p = 0.0001). The median ppOS (Figure 2D) of these groups was 5.2 versus 1.4 months (95% CI: 3.8-7.4, 0.6-3.4; Log-Rank p = 0.002), respectively. Comparing both groups in a univariate survival analysis for several of the identified prognostic markers (see Table 5), stopping vemurafenib upon progression was significantly associated with a shorter ppOS (HR 2.16; 95% CI: 1.30, 3.57; p = 0.002), as was ECOG performance status of 2 or 3, the presence of brain metastases and the serum LDH levels of 251-500 U/L and > 500 U/L. Male gender and M-stage (M1b and M1c) were also associated with a shorter ppOS, but this was not statistically significant (p > 0.05). No additional toxicities were seen in the TBP group. To decrease the possibility that the ppOS benefit evolves solely from imbalances within the cohorts, a multivariate Cox regression analysis was performed adjusting for the identified imbalances in the cohorts, such as: age, performance status, serum LDH level, M-stage and presence of brain metastases, since these are known prognostic factors for melanoma survival. Applying this analysis stopping treatment at time of progression was independently and still significantly associated with shorter ppOS (Table 6, HR 1.92; 95% CI: 1.04, 3.55; p = 0.04). Serum LDH levels higher than 500 U/L and the presence of brain metastases were also significantly associated with shorter ppOS, but not M-stage M1c and a serum LDH level between 251 and 500 U/L. It is noteworthy that the HR for TBP was hardly altered when comparing univariate with multivariate analysis (HR 2.16 versus HR 1.92).

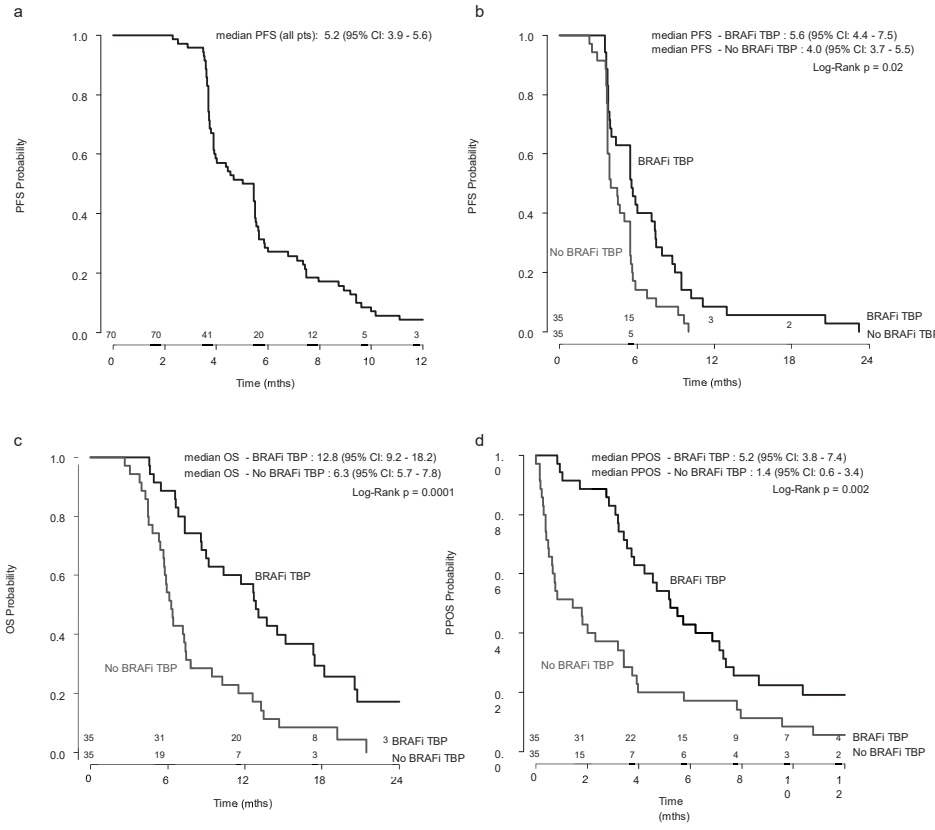


Figure 2. Kaplan-Meier curves of progression-free and overall survival in months

(a) Progression-free survival curve of total sample, (b) progression-free survival curve categorized by patients BRAFi TBP and no BRAFi TBP, (c) overall survival curve categorized by patients BRAFi TBP and no BRAFi TBP, (d) overall survival curve from the time of progression of disease. Numbers above the time-line represent the patients who are at risk at that time.

Abbreviations: BRAFi, BRAF inhibitor; OS, overall survival; PFS, progression-free survival; PPOS, post-progression overall survival; TBP, treatment beyond progression.

Sensitivity analysis was performed to see whether the number of days of BRAFi TBP used to define the cohort receiving TBP would influence overall survival from the date of progression of disease. Overall survival remained statistically different for the two groups when defining BRAFi TBP as treatment > 28 days ($p < 0.001$), > 21 days ($p < 0.001$), > 14 days ($p < 0.001$), > 7 days ($p < 0.001$) and > 0 days ($p < 0.001$). We also analyzed cost implementation of TBP. In The Netherlands one vemurafenib tablet of 240mg costs €40. This would add up to €320 a day for full dose vemurafenib. Based on a median ppOS of 5.2 months in the TBP group, TBP would add an additional "cost" of approximately €48,000-.

Table 5. Univariate analysis of post-progression overall survival (n = 70)

	Univariate analysis		
	HR	**95% CI**	**p value**
Treatment with vemurafenib			
TBP	**1**		
No TBP	**2.16**	**1.30 - 3.57**	**0.003**
Age			
Per year	**1.00**	**0.98 - 1.02**	**0.76**
ECOG performance status			
0-1	**1**		
2-3	**3.58**	**1.96 - 6.51**	**< 0.0001**
Lactate dehydrogenase			
0-250 U/L	**1**		
251-500 U/L	**1.26**	**0.68 - 2.34**	**0.460**
> 500 U/L	**5.36**	**2.50 - 11.47**	**< 0.0001**
M-stage			
M1a	**1**		
M1b	**1.97**	**0.37 - 10.85**	**0.434**
M1c	**1.71**	**0.42 - 7.01**	**0.457**
Brain metastases			
No	**1**		
Yes	**1.67**	**1.00 - 2.77**	**0.05**

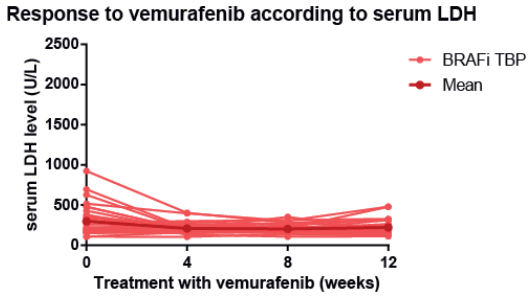
Abbreviations: CI, Confidence Interval; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, Hazard Ratio; TBP, treatment beyond progression;

Table 6. Multivariate analysis of post-progression overall survival (n = 70)

	Multivariate Cox regression analysis		
	HR	**95% CI**	**p value**
Treatment with vemurafenib			
TBP	**1**		
No TBP	**1.92**	**1.04 - 3.55**	**0.04**
Age			
Per year	**1.02**	**1 - 1.04**	**0.11**
ECOG performance status			
0-1	**1**		
2-3	**1.65**	**0.79 - 3.47**	**0.18**
Lactate dehydrogenase			
0-250 U/L	**1**		
251-500 U/L	**0.95**	**0.48 - 1.87**	**0.88**
> 500 U/L	**3.85**	**1.63 - 9.07**	**0.002**
M-stage			
M1a	**1**		
M1b	**2.39**	**0.4 - 14.2**	**0.34**
M1c	**0.7**	**0.15 - 3.18**	**0.64**
Brain metastases			
No	**1**		
Yes	**2.39**	**1.25 - 4.59**	**0.01**

Abbreviations: CI, Confidence Interval; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, Hazard Ratio; TBP, treatment beyond progression;

a



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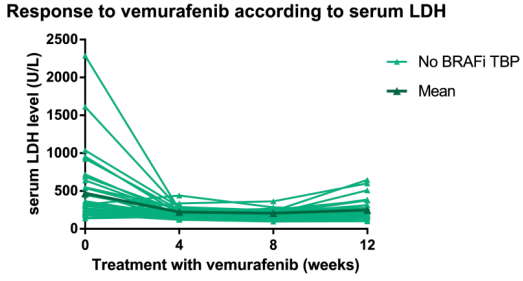

Figure 3. Serum LDH levels during treatment with vemurafenib
(a) Serum LDH levels of BRAFi TBP
(b) Serum LDH levels of no BRAFi TBP
Abbreviations: BRAFi, BRAF inhibitor; LDH, lactate dehydrogenase; TBP, treatment beyond progression.

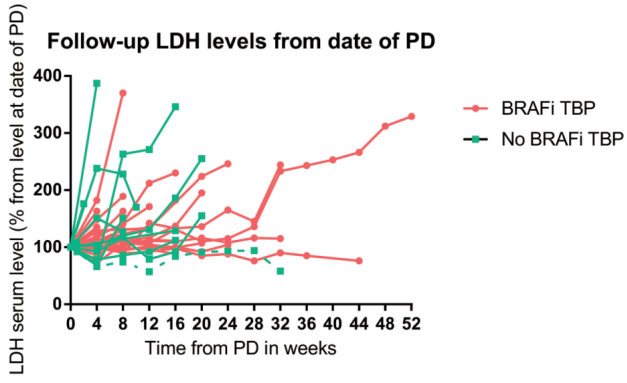

Figure 4.
Abbreviations: BRAFi, BRAF inhibitor; LDH, lactate dehydrogenase; PD, progression of disease; TBP, treatment beyond progression.

DISCUSSION

Although vemurafenib and dabrafenib have revolutionized the treatment of *BRAF*-mutated melanoma, early drug resistance and subsequent disease progression hamper long-term benefit for these patients [24, 27]. Traditionally, treatment is discontinued once progression is documented. This is especially true for classical therapies like chemotherapy with cytotoxic drugs, and this was similarly implemented in the vemurafenib versus dacarbazine phase 3 trial and initially in the Global Safety Study [24, 28, 29]. However, in the era of immunotherapy and targeted therapy, this strategy may need revision [15, 17, 18, 20, 21]. It was our clinical observation in patients treated with vemurafenib that discontinuation oftentimes lead to accelerated disease progression. Therefore, we switched our strategy and kept patients on vemurafenib (after permission of the EAP study monitor) despite progression.

In this retrospective and exploratory analysis presented here, we investigated whether BRAFi TBP could be beneficial for *BRAF* V600 mutated melanoma patients treated with vemurafenib, who initially responded to treatment. We found that BRAFi TBP was, in a multivariate analysis, significantly and independently associated with a relative reduction of nearly 50% in the risk of death, leading to a prolonged median OS after progression of 5.2 months as compared to 1.4 months in the group that stopped treatment. These data are in line with data observed for treatment with dabrafenib, the second recently approved selective BRAF inhibitor [12].

Our findings correspond also with those from other studies, which have investigated treatment beyond disease progression with targeted therapies in other malignancies [15, 17, 18, 21, 30]. For example, TBP with bevacizumab in patients with metastatic colorectal carcinoma and trastuzumab in patients with breast cancer improved OS [15, 18]. Similar results have also been found for NSCLC patients treated with EGFR inhibitors [21]. A recently published article by Chan et al. analyzing the effects of extended BRAF inhibition after progression of disease in patients with metastatic melanoma, discovered a prolonged overall survival even after adjusting for potential prognostic factors [31]. Yet other preclinical data, using xenograft models, suggest a possible adverse effect of continued BRAFi TBP. A study by Hartsough et al. discovered that growth and signaling of *in vivo* and *in vitro* derived RAF inhibitor-resistant cell lines that expressed *BRAF* V600E splice variants grew more efficiently in the presence of a BRAFi compared to without the inhibitor [32]. Another study by Thakur et al. showed that vemurafenib-resistant melanoma become drug dependent for their continued proliferation. Stopping vemurafenib treatment here led to regression of drug-resistant tumors [33]. These data, however, do need validation in humans. Furthermore, other possible BRAFi resistance mechanisms may not have

these effects on continued BRAFi TBP [34]. Our analysis in melanoma patients here does not support these findings from animal models proposing treatment discontinuation to be more beneficial. Furthermore, we did not observe any patient showing spontaneous regression after stop of BRAFi treatment.

While BRAFi treatment is showing impressive results regarding objective response rate (ORR), unfortunately there does not appear to be a plateau in overall survival as is seen with immunotherapy [35-37]. We therefore believe that TBP with a BRAFi should be reserved as a last line treatment, or should be considered as first line treatment in patients with high tumor burden, who most likely do not benefit from immunotherapy at all [38]. Treatment beyond progression will add additional costs to the health budget, but if we are able to select patients more carefully that will benefit from TBP, an additional of € 48,000.- for a median OS benefit, may still be worthwhile. Perhaps that on the basis of emerging technologies, such as cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA), we will be able to select patients that fit the group benefitting from TBP better, however further research is needed.

We are aware of the retrospective character of our analysis that might have been biased, not only by the small sample size, but also by the physicians' decision regarding continuation or discontinuation of vemurafenib depending on patients' choice, site of disease progression or treatment possibilities beyond progression. Although no variation between the groups was found in the number of patients that received subsequent treatment with, for example, ipilimumab, the physicians' decision has clearly led to imbalances between the two groups in other patients' characteristics, such as a significant difference in ECOG performance status. Also a significant difference in PFS was seen between the two groups. To minimize selection bias we conducted a sensitivity analysis that still showed a significant difference in overall survival when patients, who initially received BRAFi TBP, but who deteriorated within one month of treatment, were excluded. To analyze the possible difference in tumor biology between the two groups we compared changes of LDH upon treatment with vemurafenib. LDH has been identified as a prognostic factor and is thought to correlate with tumor metabolism [26, 39-41]. In both groups we found a normalization of the mean LDH upon vemurafenib treatment (mean serum LDH at week 8 was 203 U/L in the group treated beyond progression versus 207 U/L in the control group, $p = 0.453$) indicating no differences in the changes of tumor metabolism upon treatment (Figure 3). Interestingly baseline LDH was higher in the no BRAFi TBP group (mean LDH 470 U/L versus 311 U/L, $p = 0.066$), representing possibly a higher tumor load at treatment initiation. Considering these prognostic factor imbalances, however, this did not reduce the strong HR observed for BRAFi TBP in the multivariate analysis indicating that the imbalances in our groups had only a minor effect on the HR for BRAFi TBP.

Only a well controlled and randomized setting could provide better balanced groups and prove the benefit of treatment beyond progression in a completely unbiased setting. This study, however, would be ethically very challenging and therefore not feasible.

Since we clinically observed that once patients stopped vemurafenib, they tended to have an accelerated course of disease, we compared the serum LDH levels after progression of patients stopping vemurafenib with that of patients continuing the treatment. No significant difference was found in the rate of increase of LDH levels after stopping vemurafenib between these groups of patients (Figure 4). Since LDH levels have been considered a measurement of tumor load, these data suggest that upon stopping vemurafenib at progression, changes in LDH levels are an insufficient predictor of progressive disease.

While pretreatment serum LDH levels are prognostic factors for patients with metastatic melanoma, serum LDH levels can indicate the tumor response to vemurafenib in patients with metastatic melanoma [26, 39, 41]. We found no significant differences in LDH decrease upon treatment to vemurafenib, pointing towards similar tumor biology and thus similar initial response to selective BRAFi in the two groups. However, we cannot rule out that the lack of a difference in LDH decline was the result of the small sample size.

Our data suggest that BRAFi TBP can benefit melanoma patients, who initially responded to treatment. In the light of lack of alternative treatment options, which is not uncommon for these patients, our data suggest that BRAFi TBP with vemurafenib could be considered. A retrospective subgroup analysis of the global safety study cohort, could confirm these results, or at least give us more insight information [22]. In addition quality of life analyses should be performed. Identification of biomarkers to identify patients that benefit from TBP could round-up such analyses.

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Chapter 7

Tumor-infiltrating lymphocytes for the treatment of metastatic cancer

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ABSTRACT

Over the past few years melanoma incidence has been rising steadily, resulting in an increase in melanoma related mortality. Until recently, therapeutic options for metastatic melanoma were scarce. Chemotherapy and, in some countries, IL-2 were the only registered treatment modalities. In the last five years, treatment with immunotherapy (anti CTLA-4, anti PD-1, or the combination of these antibodies) has shown very promising results and was able to improve survival in patients with metastatic melanoma. Adoptive cell therapy using tumor-infiltrating lymphocytes is yet another, but highly promising, immunotherapeutic strategy for patients with metastatic melanoma. This review will discuss the development of TIL as a treatment option for melanoma, its mode of action and simplification over time, and the possibilities to expand this therapy to other types of cancer. Also the future directions of TIL based therapies will be highlighted.

INTRODUCTION

In 1863 Rudolf Virchow described the presence of lymphoid cells in neoplastic tissue and hypothesized a connection between inflammation and cancer [1]. Over the past two decades, clear correlations have been found between the presence of lymphocytic infiltrates within tumors and patients' clinical outcome in several tumor types, including metastatic melanoma, ovarian cancer, colorectal cancer and breast cancer subtypes [2-6]. The first attempts to isolate and characterize the lymphoid cells in cancerous tissue dates back to the 1970-ies and revealed that many tumor tissues contained lymphocytes [7, 8]. Pioneering work in this field of research has been performed by Dr. Steven Rosenberg from the Surgery Branch (SB) of the National Institutes of Health (NIH), Bethesda, Maryland. Rosenberg and colleagues started by growing tumor-infiltrating lymphocytes (TIL) from multiple murine tumors and demonstrated antitumor activity of these TILs *in vivo* [9]. In a murine sarcoma model, infusion of TIL in combination with T cell growth factor interleukin-2 (IL-2), appeared to be 50-100 times more effective in killing tumor cells than Lymphokine-Activated Killer (LAK) cells, that were generated by culturing peripheral blood lymphocytes in the presence of high concentrations of IL-2 [10]. Importantly, TIL cultured from human tumors were also able to lyse autologous but not allogeneic tumor cells in a major histocompatibility complex (MHC) dependent fashion in the majority of cases. This observation pointed towards some patient-specificity of this treatment, while this was lacking completely in LAK cell therapy [11]. In a first TIL pilot study twelve patients with metastatic cancer were treated with TIL, with or without the chemotherapeutic agent cyclophosphamide and IL-2 [12]. Two partial responses were observed, one in a patient with melanoma and one in a patient with renal cell carcinoma. Both patients received cyclophosphamide prior to TIL infusion. This was the first indication that TIL therapy could induce clinical responses in patients with metastatic cancer and formed the basis for further studies, which will be discussed in this review.

During the past decade a much better understanding of the working mechanism of TIL therapy has been gained, especially regarding the role of lymphodepleting conditioning of the host, the role of interleukin-2 as a survival factor for the infused TIL, the optimal quality and quantity of the infused cells and their antigen recognition pattern. In addition, although growing TIL was for a long time only successful in metastatic melanoma, the current protocols of TIL outgrowth are now also being explored in other types of cancer as well. These aspects and future developments will be discussed here.

TIL therapy for metastatic melanoma

Since the first clinical trial with TIL therapy by Rosenberg et al., a series of phase I/II clinical trials have shown that infusion of TIL combined with lymphodepleting preconditioning

and followed by high dose bolus infusional IL-2 can mediate objective responses in patients with metastatic melanoma [13-19]. Originally, the protocol consisted of a metastasectomy of one or more melanoma lesions. A total size of around 3 cm in diameter was required to be able to successfully grow TIL from these lesions. These resected melanomas were subsequently fragmented into microcultures in the presence of IL-2. Once enough TIL were grown from these cultures, TIL were tested for recognition of autologous melanoma cells (usually melanoma cell lines or freshly frozen tumor digest), and if not available, reactivity to a panel of human leukocyte antigen (HLA) matched allogeneic melanoma cell lines. Readout was the measurement of interferon-γ (IFN) secreted in the medium using an IFN-γ enzyme-linked immunosorbent assay (ELISA). Only those cultures containing melanoma-reactive TIL were further propagated and rapidly expanded by stimulation with soluble anti-CD3 monoclonal antibody, high concentration of IL-2 (6,000 IU/ml) and irradiated allogeneic or autologous feeder cells. Starting with approximately 50 x 10^6 TIL, these numbers were expanded in a 14-day time period to 1-20 x 10^{10} $CD3^+$ TIL. After concentration of the cells to a 200-300 ml suspension, the product was ready for infusion. It was convincingly shown that TILs selected for reactivity towards autologous melanoma cells displayed high functional activity in metastatic melanoma patients, with ORR varying between 34% to 72% of treated patients some of whom developed a long-lasting complete remission, however, there were some important drawbacks associated with this elaborate TIL production protocol [13, 16, 17]. First, the selection of TIL for reactivity against autologous melanoma required the presence of an autologous melanoma cell line. With a success rate for growing cell lines from patient material of less than 50%, the selection step on autologous tumor could not be done in at least half of the patients [20]. Secondly, as only a fraction of cultures contained tumor-reactive TILs, the total culture time to obtain enough cells for initiating rapid expansion (200 x 10^6 TIL) was long. The risk for these refractory melanoma patients to rapidly progress up to a stage that TIL therapy was no longer considered beneficial, increased with longer culture time. Thirdly, longer culture time also translated into obtaining TIL with a more terminally differentiated phenotype, decreasing their capacity to persist *in vivo* after infusion [21, 22]. Together with the inability to grow TIL from 20-25% of metastatic melanoma patients, the accumulative dropout rate amounted to 70% or more of patients that could not be treated with TIL in these early studies.

In their first clinical study with these so-called "selected TILs" Rosenberg et al. treated 86 metastatic melanoma patients, of whom 57 received a single dose of 25 mg/kg cyclophosphamide as a lymphodepleting regiment, followed by infusion of selected TIL and high-dose intravenous bolus IL-2 [13]. The overall ORR in this clinical trial was 34%. Significant differences in overall ORR were noted in patients who were treated with TIL from younger

cultures (p = 0.0001), TIL with shorter doubling times (p = 0.03) and TIL that exhibited higher lytic activity against autologous tumor targets (p = 0.0008) **(Figure 1).**

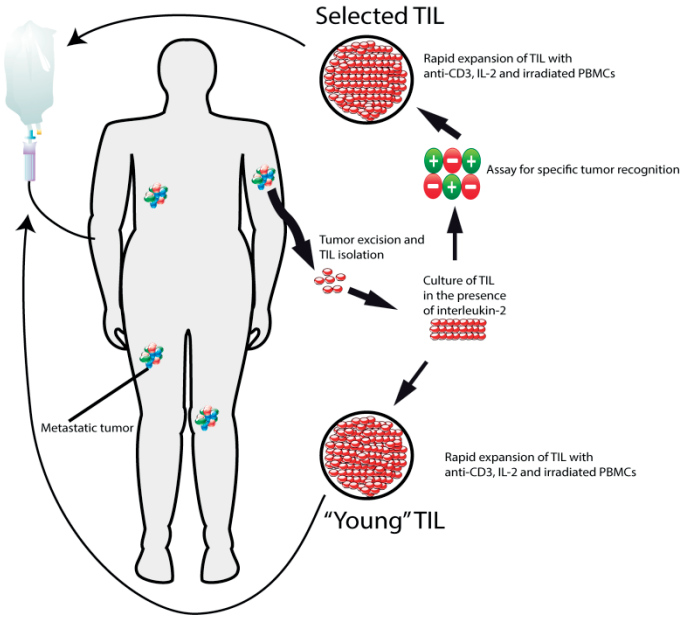

Figure 1. Schematic overview of the process for adoptive cell transfer of tumor-infiltrating lymphocytes.

After excision the melanoma metastasis is digested into a single cell suspension in 24 well plates or fragmented. These suspensions/fragments are then cultured in the presence of IL-2. In earlier days (selected TIL) cultures were tested for recognition of autologous melanoma cells (usually melanoma cell lines or freshly frozen tumor digest, and if not available a panel of HLA-matched allogeneic melanoma cell lines), by measuring IFN-γ secreted in the medium using an IFN-γ ELISA. In the "young" TIL approach this selection step for tumor reactivity has been omitted. TIL cultures are then expanded to treatment levels by stimulation with soluble anti-CD3 monoclonal antibody and, high concentration of IL-2 and irradiated allogeneic feeder cells. After concentration the product is infused in the previously lymphodepleted host.

In 2008 Dudley et al. described three cohorts of patients with metastatic melanoma treated with selected TIL in combination with different lymphodepleting regimens [14, 17]. Lymphodepleting regimens consisted of "standard" non-myeloablative (NMA) chemotherapy with cyclophosphamide and fludarabine (43 patients), or NMA chemotherapy with cyclophosphamide and fludarabine (over five instead of seven days) plus a single fraction of 2 Gray (Gy) TBI (25 patients). The third cohort of patients received the same NMA regimen as the second cohort, but instead of 2 Gy TBI, patients in this cohort received 12

Gy TBI; 2 Gy twice a day for three days (25 patients). All patients received high-dose bolus IL-2 t.i.d. to tolerance. The ORR for all 93 patients was 56%. NMA chemotherapy alone showed an ORR of 49%, when 2 or 12 Gy TBI was added, the response rates were 52% and 72%, respectively. Twenty complete remissions were seen in this clinical trial. A significant difference in ORR was noted in patients receiving less IL-2 ($p < 0.001$), patients receiving TIL with longer telomeres and larger fractions of $CD8^+CD27^+$ cells ($p < 0.001$). Despite the differences seen in ORR, there appeared to be no significant difference in overall survival when comparing the three groups ($p = 0.13$). A separate early clinical trial was performed at the Moffitt Cancer Center, Tampa, Florida, with 19 patients of whom 13 were treated with selected TIL. The ORR was 38% for treated patients and 26% for the total group [23].

These clinical data nicely illustrate the reproducible efficacy of TIL therapy for metastatic melanoma. However, as little is known about the exact dropout rate of patients that were intended to be treated, these exciting response rates were somewhat misleading. The studies pointed clearly towards the benefits of creating a TIL infusion product in the shortest possible culture time and infusion of as many as possible TIL, displaying a more central memory phenotype (CD27 and CD28 positive) and long telomeres. In order to fulfill these goals and decrease the dropout rate, the investigators at the SB amended the TIL production protocol by leaving out the selection step. Without the selection step for tumor-reactivity, the culture time was decreased by on average three weeks, rendering the cells 'younger', hence the name 'young TIL protocol. As a result of this modification at least 50% of patients, who were referred for TIL therapy, could be treated.

The first clinical trial, in which patients with metastatic melanoma were treated with young TIL, also included a CD8 enrichment step [18]. This was considered prudent because of the risk that possibly Tregs were infused as well, if bulk TIL were given. In this trial, 122 patients with metastatic melanoma were enrolled, however only 56 patients could be treated, mainly due to either inability to grow TIL from tumor digests (17%), disease progression prior to TIL infusion (16%), or no evaluable disease after metastasectomy (11%). Although the dropout rate was still high (50%), this was substantially less compared to the delivery of selected TIL. The ORR for all treated patients in this trial was 54%. Within the group of patients that received NMA TIL an ORR of 58% was observed, compared to 48% for patients treated with NMA + 6 Gy TBI. The ORR for all 122 enrolled (intention to treat) patients was 25%.

The clinical protocol of using unselected young TIL in combination with NMA and high dose IL-2 was subsequently implemented in TIL trials at other centers in and outside the US. The results from these trials are summarized here.

At the Ella Institute in Tel Aviv, Israel, 55 patients with metastatic melanoma, who had received at least prior high dose IL-2, were enrolled in a phase II clinical trial with young TIL [24]. Thirty-two patients received TIL infusion. The dropout rate was 42%, mostly due to development of brain metastases, rapid disease progression, and inability to grow TIL. The ORR for patients that had received TIL infusion was 47%, including four patients with a complete response (CR), whereas the ORR for the total cohort of 55 patients was 27%. These results were very much in line with the outcomes observed in the study with CD8-enriched TIL at the SB. Also in agreement with prior studies was the finding of a significant correlation between patients receiving TIL with a shorter culture time ($p = 0.0008$), higher number of infused cells ($p = 0.0251$), or TIL cultures with a higher percentage of $CD8^+$ T cells ($p = 0.0144$) and outcome (ORR).

This study was updated recently and reported on 80 patients, of whom 57 were treated with young TIL following NMA with cyclophosphamide and fludarabine and high dose bolus IL-2 following TIL infusion [25]. In the intention-to-treat analysis the ORR was 29% and for the treated group 40%. The total number of complete responders was 5%. The 3-year overall survival of responding patients was 78%.

In another trial conducted at the MD Anderson Cancer Center, 31 patients with metastatic melanoma were treated with young TIL [26]. The biggest difference relative to the Ella Institute protocol was a second course of high-dose (HD) IL-2 three weeks after TIL infusion. ORR for the 31 treated patients was 42%. Significant differences in ORR were seen in patients receiving more TIL ($p = 0.0003$), patients receiving a higher percentage of $CD8^+$ cells ($p = 0.001$) and patients receiving a higher absolute number of $CD8^+$ cells ($p = 0.0003$). Two patients developed a complete response.

At the Herlev Hospital in Copenhagen, Denmark, we treated patients with TIL in two sequential studies. One pilot study in which NMA TIL was combined with low dose IL-2 and a second phase II study with decrescendo IL-2 dosing (see section on IL-2). Thirty-three patients were enrolled in the phase II trial of whom 25 were treated with TIL. Ten of 24 evaluable patients obtained an objective response, of which 3 CR (R. Andersen, manuscript submitted).

In 2013 Dudley et al. reported the results of a randomized controlled phase II clinical trial in patients with metastatic melanoma who were randomized to receive either $CD8^+$ enriched young TIL or unselected young TIL [19]. Hundred and one patients were enrolled in this clinical trial of whom 69 were actually treated with TIL. Of these 35 patients received CD8-enriched TIL and 34 received unselected young TIL. ORR for the two arms

of the study were 20% and 35% respectively, although this difference was not statistically significant due to the small number of patients that were enrolled in this study.

These selected clinical trials utilized young TIL for the treatment of metastatic melanoma. Although the treatment protocols were not completely equal (use of TBI next to NMA, different schedules of IL-2, CD8-enrichment), the outcome of these trials conveyed very similar messages. When combining the 3 largest studies a total of 336 patients were enrolled. Of these 207 patients were actually treated with TIL, resulting in a dropout rate of 38% of patients, mostly due to rapid disease progression, development of symptomatic brain metastases, inability to generate TIL or due to withdrawal of informed consent. An objective response was seen in 82 patients, or 40% of treated patients and 24% of all enrolled patients. In all four clinical trials combined 18 complete responses were seen, this amounts to 9% of all treated patients, or 5% of all enrolled patients (see Table 1).

Role of lymphodepletion

Several mouse models have demonstrated that conditioning of the host by use of chemotherapy or total body irradiation (TBI) improved the response rate of adoptive T cell therapy. Berendt and North were the first to point out that immunosuppressive T cells from the host could prevent complete eradication of established transplanted tumors by adoptive T cell therapy [31]. Thus, only hosts that were T cell deficient by prior thymectomy demonstrated tumor rejection. Similarly, the use of cyclophosphamide and TBI in conjunction with adoptive cell therapy appeared much more effective in comparison to non-pretreated mice [32, 33]. Also in patients with metastatic cancer, lymphodepleting host conditioning resulted in high objective response rates (ORR) upon adoptive cell transfer and durable benefit for the treated patients [15]. By studying the immunological effects of host lymphodepletion in murine models, several mechanisms of action have been suggested. First, by inducing a temporary lymphopenic state in the host the remaining peripheral lymphocytes will restore the original lymphocyte pool by a process called homeostatic expansion. Under these conditions, the infused syngeneic lymphocytes were more likely to expand and engraft *in vivo*. Second, lymphodepletion could cause a decrease in competition with endogenous T cells for antigen-presenting cell interaction. Recently, Gattinoni et al., demonstrated in a murine B16 melanoma model that infusion of gp100-specific pmel-1 T cells followed by IL-2 was much more effective in non-lethally irradiated animals than in non-irradiated mice. Induction of lymphopenia did not result in increased expansion of adoptively transferred pmel-1 T cells, but rendered these cells functionally much more active. This phenomenon could be explained by the depletion of regulatory and immunosuppressive $CD4^+$, $FoxP3^+$ regulatory T cells (Tregs), which is a potentially third effect of lymphodepletion.

Fourth, removing so-called cellular sinks, especially NK cells that highly compete with the adoptively transferred T cells for the host homeostatic cytokines IL-7 and IL-15 is considered a very important contribution of lymphodepletion on efficacy of TIL therapy. Whereas IL-7 appears to be required for the proliferation and survival of the T cells, IL-15 critically serves to maintain or improve the functional quality of the pmel-1 T cells [34]. Notably, in patients receiving lymphodepleting conditioning regimens the serum concentrations of IL-7 and IL-15 also increased [17].

In patients with metastatic melanoma, lymphodepleting chemotherapy consisting of cyclophosphamide and fludarabine induces a temporary lympho- and leukopenic state lasting around 5-10 days. For bone marrow recovery $CD34^+$ peripheral bone marrow stem cell support is not required. Dudley et al. examined whether intensifying the lymphodepletion by adding TBI to the non-myeloablative chemotherapy (NMA) regimen, would improve the outcome of TIL treated patients [17]. Two cohorts of 25 patients each were treated either with cyclophosphamide/fludarabine plus 2 Gy TBI, or 12 Gy TBI. In both groups bone marrow recovery was supported by autologous peripheral blood stem cell transplantation. Compared to a cohort of patients treated with chemotherapy alone (ORR 48.8%), adding TBI resulted in ORR of 52% and 72% respectively for 2 Gy and 12 Gy TBI. As this was not a randomized controlled trial, these differences in outcome could be explained by variation in patient selection, however this outcome warranted direct comparison in a randomized controlled trial (RCT). This clinical trial (ClinicalTrials.gov Identifier: NCT01319565) is still ongoing, but preliminary results presented so far fail to show a difference in clinical outcome between patients treated with chemotherapy compared to chemotherapy plus 12 Gy TBI (Rosenberg, personal communication).

In summary, conditioning by depletion of lymphocytes and NK cells appears to be an important component in the success of TIL therapy for metastatic melanoma, through depletion of immunosuppressive cells from the host and tumor micro-environment and removal of cellular sinks for homeostatic cytokines IL-7 and IL-15. So far, the necessity for increased lymphodepletion has not clearly been demonstrated. Obviously, a more stringent myeloablative conditioning regimen, requiring autologous $CD34^+$ stem cell support, would complicate a wider application of TIL therapy considerably.

Interleukin-2 dosing schedule

In the original TIL treatment regimen published by Rosenberg et al. a high-dose (HD) bolus IL-2 schedule of 720.000 IU/kg i.v. every 8 hours was initiated immediately after TIL-infusion and continued until treatment limiting toxicity [14]. This classical HD IL-2 schedule has been used as standard of care for treatment of metastatic melanoma for

Table 1. Studies evaluating the effect of tumor-infiltrating lymphocytes in patients with metastatic melanoma

Reference	TIL production	Culture time (weeks)	Enrolled patients	Treated patients (%)	Reason dropout		
					PD or development sBM	No TIL	Other
[13]	Selected	-	86	86 (100)	-	-	-
[27]	Selected	5-7 (without REP)	41	43 (2 patients received multiple treatments)	-	-	-
[14, 17]	Selected	5-8	93	93(100)	-	-	-
[18]	CD8+ enriched "young"	4-5	122	53 (43) + 3 additional patients from prior resections	20	21	28
[28]	Selected	7-8 (including REP)	11	6 (55)	4	1	-
[29]	Selected	6 (not including REP)	24	24 (100)	-	-	-
[23]	Selected	8-10 (including REP)	19	13 (68)	4	1	1 (SAE during chemotherapy)

Lymphodepleting chemotherapy regimen	IL-2 regimen	Response according to RECIST		
		OR (n)	% OR (enrolled patients)	% OR (treated patients)
57 received Cy (25 mg/kg) as single infusion	720,000 IU/kg t.i.d. to tolerance Repeated after 21 days	29	34	34
16 patients received Cy (25 mg/kg) as single infusion	720,000 IU/kg t.i.d. to tolerance, or 216,000 IU/kg and IFN-alpha $3x10^6$ U/m^2 t.i.d. to tolerance	9	21	21
1st cohort Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1) 2nd cohort Cy 60 mg/kg (day -6&-5) + Flu 25 mg/m^2 (day -6 through -2) + 2 Gy TBI 3rd cohort Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -7 through -3) + 2 x 2 Gy TBI per day for 3 days	720,000 IU/kg t.i.d. to tolerance Maximum of 15 doses	1st cohort 21 (5 CR, 16 PR) 2nd cohort 13 (5 CR, 8 PR) 3rd cohort 18 (10 CR, 8 PR)	1st cohort 49 2nd cohort 52 3rd cohort 72 Total: 56	1st cohort 49 2nd cohort 52 3rd cohort 72 Total: 56
1st cohort Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1) 2nd cohort Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1) + 3 x 2 Gy TBI	720,000 IU/kg t.i.d. to tolerance Maximum of 15 doses	1st cohort 19 (3 CR, 16 PR) 2nd cohort 11 (2 CR, 9 PR) Total: 30 (5 CR, 25 PR)	25	1st cohort 58 2nd cohort 48 Total: 54
Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1)	2 MIU s.c. for 14 days	2 (2 CR)	18	33
Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1)	2.4 x 10^6 units / m^2 until PD or toxicities	5 (1 CR, 4 PR)	21	21
Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1)	720,000 IU/kg t.i.d. to tolerance Maximum of 15 doses	5 (2 CR, 3 PR)	26	38

Table 1. Studies evaluating the effect of tumor-infiltrating lymphocytes in patients with metastatic melanoma (*continued*)

Reference	TIL production	Culture time (weeks)	Enrolled patients	Treated patients (%)	Reason dropout		
					PD or development sBM	No TIL	Other
[26]	"Young"	7 (including REP)	31	31 (100)	-	-	-
[19]	"Young"*	3-7	101	69 (68)	15	17	-
[25]	"Young"	4 (including REP)	80	57 (71)	11	8	3 refused
[30]	"Young"	-	33	25 (76)	7	1	0

* Either unselected "young" TIL or unselected "young" $CD8^+$-enriched TIL

Abbreviations: CR, complete remission; Cy, Cyclophosphamide; Flu, Fludarabine; Gy, Gray; IU, international unit; kg, kilogram; mg, milligram; OR, objective response; PD, progressive disease; PR, partial remission; REP, rapid expansion protocol; SAE, serious adverse even; sBM, symptomatic brain metastases; s.c., subcutaneous; t.i.d., ter in die; TIL, tumor-infiltrating lymphocytes

Lymphodepleting chemotherapy regimen	IL-2 regimen	Response according to RECIST		
		OR (n)	% OR (enrolled patients)	% OR (treated patients)
Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1)	720,000 IU/kg t.i.d. to tolerance Maximum of 15 doses Second cycle of IL-2 21 days post TIL infusion	13 (2 CR, 11 PR)	42	42
Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1)	720,000 IU/kg t.i.d. to tolerance Maximum of 15 doses	1st cohort 12 (2 CR, 10 PR) 2nd cohort 7 (3 CR, 4 PR) Total: 19 (5 CR, 14 PR)	19	28
Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1)	720,000 IU/kg t.i.d. to tolerance Maximum of 15 doses	23 (5 CR, 18 PR) 1 patient died during chemo-therapy regimen	29	40
Cy 60 mg/kg (day -7&-6) + Flu 25 mg/m^2 (day -5 through -1)	18 MIU/m^2 s.c. over 6, then 12 and then 24 hours, followed by 4.5 MIU/m^2 over 24 hours q 3 days	10 (3 CR, 7 PR) 1 patient not yet evaluated	30	40

several decades and the rationale for its use after TIL infusion is to support the continued growth and activity of the infused TIL [35].

The HD IL-2 regimen is associated with transient but severe systemic toxicity affecting multiple organ systems and restricting its use to highly specialized cancer centers with experienced clinicians and intensive care support [36]. To this end, HD IL-2 administration to patients experiencing pancytopenia after HD chemotherapy leads to a particularly vulnerable medical condition with the need of intensive monitoring and specialist care.

The requirement for repeated high doses of IL-2 in order to obtain clinical efficacy after TIL based ACT has never been documented in the clinical setting. On the contrary, data from the SB showing that patients who experienced an objective response received fewer doses of HD IL-2 as compared to non-responders, have recently questioned the administration of multiple high doses of IL-2 [37]. This might be explained by the fact that IL-2 administration significantly increased the number of Tregs with a direct correlation between the number of IL-2 doses given and reconstitution of Treg numbers in the blood and an inverse correlation between reconstitution of the Tregs and the probability of achieving an anti-tumor response [37].

At the Center for Cancer Immune Therapy, Herlev Hospital, University of Copenhagen, Denmark we have tested a low and an intermediate IL-2 dose schedule TIL based ACT. In an initial pilot study including six melanoma patients a low-dose regimen of IL-2 was used, consisting of subcutaneous (s.c.) administration of IL-2, 2 million international units (MIU)/day for 14 days. Two of these patients achieved complete and long-lasting responses [28]. Both patients experienced recurrence of a solitary metastasis (1 and 3 years after therapy), which was surgically removed and are currently free of disease more than 4 years after therapy. In a subsequent phase II trial presented at ESMO 2015, the intermediate decrescendo IL-2 schedule was used [38]. This regimen consists of five days continuous intravenous (i.v.) infusion of decreasing IL-2 doses: 18 MIU/m^2 over six, then 12, and then 24 hours followed by 4.5 MIU/m^2 over 24 hours for three days. In this study 25 patients were treated, with an ORR of approximately 40% which is comparable to what has previously been published with high dose bolus IL-2 [30]. Low-dose subcutaneous IL-2 was associated with very limited toxicity while i.v. decrescendo IL-2 led to increased, but certainly manageable toxicity, without the requirement for intensive care support.

These studies indicate that objective and durable responses can in fact be induced without the use of HD IL-2. Thus, the optimal dosing of IL-2 after TIL transfer in regard to clinical efficacy as well as toxicity requires further investigation, which may likely lead to dose reduction of IL-2 in the future. A randomized phase II trial, TIL therapy in metastatic

melanoma and IL-2 dose assessment (ClinicalTrials.gov Identifier: NCT01995344), testing HD versus low dose IL-2 is planned at The Christie Hospital NHS Foundation Trust, Manchester, United Kingdom, but is not yet recruiting patients. Another non-randomized phase II study at the SB, plans to assess the feasibility of TIL based ACT for melanoma without the use of IL-2 (ClinicalTrials.gov Identifier: NCT01468818).

Quality and quantity of TIL

Preclinical models on adoptive cell therapy for the treatment of cancer demonstrated the absolute requirement for $CD8^+$ T cells within the infusion product for anti-tumor efficacy. In some models, the presence of $CD4^+$ T cells was required as well [39]. In addition, the absolute numbers of transferred T cells correlated with outcome in these models, showing that infusion of more cells resulted in better tumor control. Other factors such as lymphodepletion and combination with high dose of IL-2 improved persistence of the TILs after transfer and efficacy of the treatment [40, 41]. Based on these preclinical findings, clinical trials were designed and many aspects of preclinical evidence were found in human studies as well. In clinical trials performed at the SB and other centers, correlation between ORR and absolute number of infused T cells was very consistent [24, 26]. However, a clear correlation between *in vitro* antitumor reactivity of the TIL product and clinical response has not be demonstrated, suggesting that the TIL products with the highest fold expansion might hold the "fittest" cells with the highest antitumor activity.

Infusion of a less differentiated cell population is another important factor in improving the efficacy of TIL both in preclinical models and humans [42]. As TIL, by virtue of their presence within the tumor micro-environment, are thought to be antigen experienced T cells, these cells have already gained effector function. Correlations with clinical outcome have been found for surface expression of the co-stimulatory molecules CD27 and CD28 by the infused cells, which is indicative of a less terminally differentiated phenotype [21, 22]. In a report by Tran et al., the expression of CD27 and CD28 was measured by flow cytometry in young TIL and standard TIL cultures [43]. Fourteen matched pairs of young (mean culture age of 12 days) and standard (mean culture age 25 days) TIL were generated from tumor specimens. Flow cytometry analysis demonstrated that phenotypic expression of CD27 and CD28 differed in young versus standard TIL. Young TIL had significantly higher expression of CD27 and CD28, $p < 0.00001$ and $p = 0.003$, respectively, confirming their less differentiated phenotype.

Indicative of a less differentiated T cell pool is also its proliferative capacity at the time of infusion, as determined by the length of telomeres. In several clinical studies longer telomere length was associated with ORR [13, 17, 24]. It was shown that although telomere

lengths varied widely at any given TIL age, there was an inverse correlation between culture time and mean telomere length of TIL ($p < 0.001$).

TIL products contain variable quantities of CD4+ T cells, but their role in mediating tumor regression has not been well clarified. Some studies suggested that a higher percentage of CD4+ TILs in the infusion products may be associated with worse outcomes after treatment [26, 44]. However, reports on single patient cases seem to indicate that effector CD4+ TILs may mediate antitumor effector functions [45-47]. More recently, we showed that about 50% of patients with melanoma harbor tumor-reactive CD4+ TILs. These cells can recognize MHC class II positive autologous melanoma cells but are largely monofunctional (Donia M et al., manuscript submitted). It seems therefore unlikely that, in the majority of patients, tumor-specific CD4+ T cells mediate clinical effects.

More recently, in depth phenotypic analysis comparing characteristics of $CD8^+$ TIL to peripheral blood $CD8^+$ T cells from the same patients indicated that TIL have a distinct expression pattern of co-inhibitory and co-stimulatory molecules PD-1, LAG3, TIM3 and 4-1BB (CD137) [48]. Although the level of expression varied, $CD8^+$ TIL invariably showed higher expression of these molecules compared to peripheral blood $CD8^+$ T cells. Importantly, the *in vitro* tumor-reactive T cells within TIL resided within this population. When stimulated with the autologous tumor cells, only $PD1^+$, $LAG3^+$ and $TIM3^+$ TIL showed cytolytic activity, produced IFN-γ and started to express 4-1BB (as marker of T cell activation), whereas the $PD1^-$, $LAG3^-$ and $TIM3^-$ cells failed to do so. Clonotypic frequencies measured by TCRVbeta sequencing between $PD1^+$ and $PD1^-$ $CD8^+$ TIL differed considerably, showing oligoclonal expansion within $CD8^+/PD1^+$ compared to $CD8^+/PD1^-$ TIL, reminiscent of prior antigen encounter and antigen-driven proliferation. The tumor-reactive TIL resided within the $CD8^+/PD1^+$ clonotypes. In another study by Ye et al. 4-1BB was mainly expressed on the tumor-reactive lymphocyte subset within TILs [49]. In this study, $4\text{-}1BB^+$ and $4\text{-}1BB^-$ T cells from ovarian cancer were cultured overnight in median supplemented with IL-7/IL-15. The $4\text{-}1BB^+$ and $4\text{-}1BB^-$ fractions were then cultured for 8-10 days in IL-2 and tested for reactivity against autologous tumor cells. $4\text{-}1BB^+$ TILs secreted IFN-γ in response to autologous tumor cells, whereas $4\text{-}1BB^-$ TILs did not. These results strongly suggest that pre-selection of TIL either by PD1 expression or 4-1BB prior to rapid expansion, can lead to enrichment of tumor-reactive T cells and increase the efficacy of this treatment. Not only does 4-1BB play a role in the possible selection of tumor-reactive T cells, 4-1BB co-stimulation could also be involved in improving TIL survival following ACT and potentially boost anti-tumor cytolytic activity. It is known that the majority of post-REP $CD8^+$ T cells lose the expression of the co-stimulatory molecule CD28 [50]. Furthermore, the expression of the co-stimulatory molecule CD27 is lost after stimulation of TILs with IL-2 [51]. With the loss of both CD27 and CD28 alternative co-stimulation pathways may have an important

role in maintaining TIL survival after ACT, and 4-1BB could be such a candidate. When the agonistic anti-4-1BB antibody was added during the initial tumor fragment cultures to provide 4-1BB costimulation, this resulted in an accelerated expansion of $CD8^+$ TIL. Furthermore, it also appeared that TIL expanded in the presence of anti-4-1BB antibody showed increased antitumor reactivity, as measured by INF-γ release after a 24-hour tumor cell-TIL co-culture assay [52, 53].

TIL recognition of tumor antigens

T cells recognize antigens expressed at the cell surface presented by MHC class I and II molecules. For melanoma TIL, recognition of several classes of antigens have been described. First, there are antigens derived from melanocyte differentiation antigens (MDA), especially MART-1 and gp100, but also tyrosinase and tyrosinase related peptides 1 and 2 [54-58]. In many TIL, $CD8^+$ T cells specific for MART-1 and gp100 have be found [59]. Most melanomas express MART-1 and gp100, and the fact that T cells specific for these antigens are sometimes abundantly present in TIL, at least suggest that these T cells have undergone antigen-specific expansion. As these proteins are also expressed in normal melanocytes in skin, eye and inner ear as well, one could expect that following infusion of 10^{11} TIL harboring MART-1 or gp100 specific T cells, patients would develop toxicities as a result of melanocyte destruction, such as skin rash, vitiligo, uveitis or even the Vogt-Koyanagi-Harada syndrome (uveitis, dermatitis, with also neurologic and inner ear involvement due to melanocyte destruction). Although these toxicities were indeed observed in patients treated with T cells genetically modified to express high-affinity MART-1 or gp100-specific T cell receptors, this was not the case in the many melanoma patients that have been treated with TIL, despite the (oftentimes low abundant) presence of MART-1 or gp100-specific cells, thereby perhaps questioning the relevance of these cells for melanoma rejection. A correlation between presence of these cells and outcome after TIL treatment has not been demonstrated [60].

Another class of antigens that is recognized by melanoma TIL are Cancer/Testis (C/T) gene products. These genes are normally expressed during embryogenesis and in germ cells, however are silenced in other tissues. Many tumors can start to aberrantly express these genes. One example is the melanoma antigen (MAGE), first described by Boon and colleagues, expressed on melanoma cells and other tumors, but not on normal tissue [61]. Later, many more C/T antigens were discovered, including SSX2, NY-eso-1, RAGE and SAGE [62, 63]. Some of them have sub-members, such as the MAGE antigens (MAGE-A1 through 12, MAGE-B and MAGE-C) family members, and many are expressed on a wide variety of different tumor histologies. In a recently published study, we carefully examined the frequency of $CD8^+$ T cells specific for previously described C/T epitopes within melanoma TIL infusion products from the SB and the Ella Institute. The screen,

which utilized soluble peptide-MHC multimers, (HLA-A*0201 harboring known antigenic peptides from C/T antigens) in a flow-cytometry based combinatorial encoding strategy [64, 65], revealed that C/T antigen specific T cells can oftentimes be found, although in the majority of patients tested, the frequency of these cells was rather low, seldom higher than 0.1% of $CD8^+$ TIL [60]. That C/T antigen specific T cells can result in tumor rejection, was endorsed by an adoptive T cell transfer study using peripheral blood T cells genetically equipped with a NY-eso-1 specific TCR [66]. The role of C/T antigen specific TIL in tumor rejection is not yet fully appreciated, and may differ between tumor types.

Next to expressing C/T antigens, tumors may also overexpress proteins that give rise to antigen-specific T cell responses. One example is Meloe-1, encoded by a gene that is overexpressed as a result of epigenetic changes in the tumor [67, 68]. The aforementioned screen of melanoma TIL infusion products also included known overexpressed antigens. $CD8^+$ T cells specific for these antigens were present within TIL coming from several patients. Again the frequency of these T cells was generally very low.

With current DNA technologies readily available, full exome sequencing of tumor derived DNA has become feasible in a limited period of time and to affordable costs. The Welcome Trust Sanger Institute recently published the results of high fidelity DNA sequencing of many human tumors and revealed the mutational load within these tumors [69]. On average, melanomas were found to contain the highest number of somatic mutations per megabase of DNA, followed by NSCLC, bladder cancer, stomach and esophageal cancer, whereas leukemias harbor only few mutations Already several decades ago, melanoma derived T cells specific for mutated antigens such as CDK4 and β-catenin were described [70, 71], however their role as tumor rejection antigens has largely been ignored as these mutations are patient specific and rare. To identify potential neo-epitopes, whole exome DNA sequence data of tumor and matching healthy cells need to be aligned in order to detect patient-specific mutations. RNA expression data is used to subsequently assess whether a mutated gene is transcribed and its gene-product potentially expressed on the tumor cell surface. Several approaches can be followed to assess whether the T-cell based immune system is able to recognize and respond to these mutated antigens. One such approach followed by the SB utilizes synthesis of minigenes encoding fragments corresponding to the mutation flanked on both sides by four amino acids. These minigenes were transiently transfected into COS-7 cells for stimulation of TIL [72]. A different approach followed by our group at the Netherlands Cancer Institute (NKI) utilized peptide-MHC binding algorithms to predict potential epitopes around these mutations for the different HLA molecules of the patients, followed by the generation of peptide-MHC multimers and screening of TIL for the presence of neo-antigen specific $CD8^+$ T cells [73]. Using a different approach, we were able to screen for neo-antigen specific $CD4^+$ T cells as well [74]. In

the vast majority of patients, both approaches led to the discovery of neo-antigen specific T cell responses within TIL products. In most cases, the frequency of TIL reactive against mutated antigens appeared higher than what was previously observed for other antigen classes. However, despite the high number of nonsynonymous somatic DNA mutations found in melanoma, only very few appear to lead to a neo-antigen specific T cell response. This may be explained by 1.) not all DNA mutations are in expressed genes, 2.) mutated proteins need to be properly processed to generate class I binding epitopes 3.) the TCR repertoire needs to cover these potential neoantigens, 4.) our technical set-up may be far from optimal (incomplete RNA seq, imperfect prediction algorithms for binding to different HLA molecules). Importantly, the few neoantigen specific T cells responses found per patient so far are highly unique for every patient, indicating that the induction of a T cell response against mutated antigens appears to be a random process that can best be explained by a probabilistic lottery model [75].

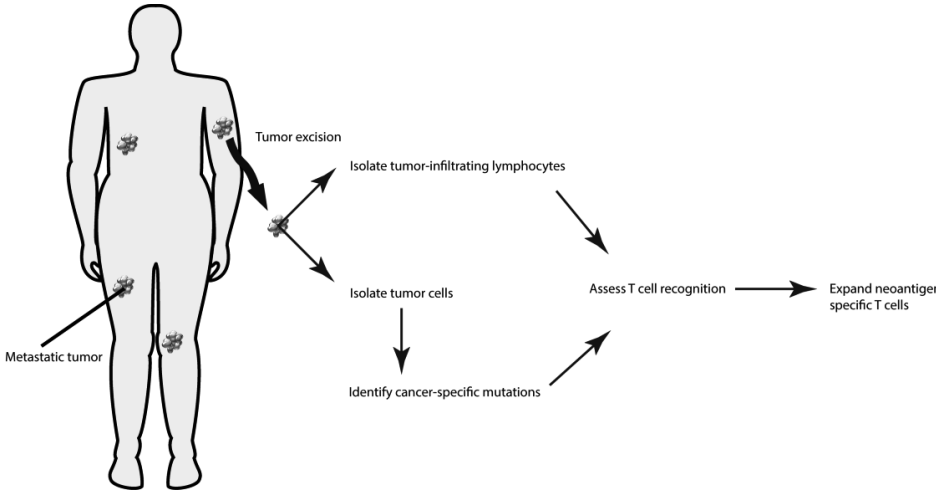

Figure 2. Schematic overview of the possible selection of neo-antigen specific TIL

Excised metastatic melanoma is analyzed for cancer-specific mutations. Resulting epitopes are used to identify neo-antigen specific T cell responses within T cell populations. The neo-antigen specific T cells are then further expanded and infused into the previously lymphodepleted patient.

In conclusion, within melanoma TIL reside T cells, both $CD4^+$ and $CD8^+$ that recognize tumor antigens. These antigens can be derived from MDA, C/T genes, overexpressed genes and mutated genes. Immunological tolerance is likely lacking for epitopes coming from mutated genes, which could result in higher affinity T cells than those specific for most

other antigens. Whether these T cells are in general functionally superior still remains to be demonstrated.

Beyond melanoma – TIL for other tumor types

The presence of TILs and its association with improved survival has been documented in virtually every human cancer studied [76, 77]. It therefore seemed logical to test TIL therapy for any solid tumor, depending on the ability to grow TIL that are reactive to autologous tumors.

So far, adoptive cell transfer immunotherapy with TILs has been applied with consistent success only in metastatic melanoma. Indeed, early studies in melanoma showed that TILs could be expanded *in vitro* and recognize autologous tumors, but until recently it was difficult to demonstrate similar level of tumor recognition in other tumor histologies [78, 79]. Here we describe the main advances in characterization of TIL and application of TIL therapy in other major types of solid tumors.

Cervical Cancer

Persistent infection with human papilloma viruses (HPVs) is essential in the pathogenesis of virtually all cervical cancers [80], and prophylactic HPV vaccination is now recognized as a standard procedure for the prevention of cervical cancer and other HPV-associated diseases [81]. However, despite its immunogenicity and encouraging recent results in patients with high-grade cervical intraepithelial neoplasia [82], an effective therapeutic vaccine for established cervical cancer has not yet been developed. It was suggested that the relatively low magnitude of vaccine-induced immune responses, and the immune suppression mediated by large established tumors may represent a barrier to the induction of immune-mediated regression of cervical cancers with classical immunization protocols [83].

In a recent study, Stevanovic et al. reported that out of nine patients with recurrent metastatic cervical cancer treated with TIL therapy (when possible, TIL microcultures were selected for HPV E6 and E7 oncoprotein reactivity), three patients experienced an objective response including two patients with complete tumor regression lasting over one year. Interestingly, responses occurred only when reactivity to E6 and E7 HPV-related oncoproteins was demonstrated in TILs [84]. Though these findings may warrant further optimization, it is currently not known whether the T cell reactivity to E6 and E7 by itself is mediating tumor regression or, rather, whether this represents a biomarker of more potent antitumor immune responses directed towards other tumor antigens.

This pivotal study demonstrates the feasibility and efficacy of this approach in selected patients with metastatic cervical cancer, and will ensure further development of adoptive cell therapy in this malignancy.

Ovarian Cancer

In ovarian cancer, the prognostic significance of tumor-infiltrating T-cells has been known for over a decade [5] and several recent studies have confirmed and expanded on these results [85]. For instance, two recent studies from independent groups, demonstrated the presence of functional tumor-antigen specific T-cells in the tumor microenvironment of ovarian cancer [86, 87].

Through characterization of the tumor mutanome and comprehensive screening of mutation-specific T-cells obtained from the tumor microenvironment of three patients, Wick et al. demonstrated a highly specific $CD8^+$ T cell response to a nonsynonymous mutation in one patient [88]. Thus, these data demonstrate that some degree of immune surveillance to the tumor mutanome may be present in selected patients with ovarian cancer.

Encouraging clinical data from the use of TILs in ovarian cancer were already reported in the 90's [89-91]. However, based on the current knowledge of the biology of immune responses to ovarian cancer, as well as new protocols for adoptive transfer, which consistently demonstrated efficacy in melanoma, the possibility to revisit TIL therapy for ovarian cancer is highly warranted. To this end, we are currently developing optimized protocols to apply TILs in ovarian cancer.

Kidney Cancer

It has been known for decades that effective manipulation of the immune system can mediate durable complete responses in a small fraction of patients with advanced kidney cancer [92]. However, despite early demonstration that renal cell carcinoma (RCC) contain tumor-antigen specific cytotoxic T-lymphocytes [93-95], previous clinical trials with TIL therapy in RCC has been quite disappointing and, in general, it has been particularly difficult to demonstrate any tumor reactivity of RCC TIL (reviewed in [96]).

In recent years, two studies demonstrated the utilization of optimized methods for TIL manufacturing to generate high numbers of TILs with (at least to some extent) tumor reactivity.

Schachter and co-workers (Sheba Medical Center, Tel-Hashomer, Israel), who are very experienced in applying TIL therapy in metastatic melanoma, have tested the same exact

methods of TIL manufacturing that have been used with success in melanoma in RCC [97]. They demonstrated that TILs from some patients could exert antitumor functions. Indeed, in a few cases TILs secreted IFN-γ upon recognition of autologous tumors, while in other cases TILs exerted killing activity. Surprisingly, none of the TIL cultures demonstrated simultaneous killing and IFN-γ secretion [97]. The relatively low sensitivity of some assays used in this study may explain this apparent paradox. However, functional deficiencies of tumor-specific TILs in RCC have indeed been described, thus firm conclusions cannot be drawn [95].

The group of R. Hawkins (University of Manchester, UK), also with extensive experience in generating TILs from metastatic melanoma, was able to optimize the method of expansion by using anti-CD3/anti-CD28-coated paramagnetic beads. With this method, IFN-γ secretion from expanded TILs co-cultured with uncultured tumor cells was shown in about 50% of patients [98].

Despite the heterogeneous and, in some cases, conflicting results, these studies demonstrate that TIL therapy may be feasible in RCC and warrant additional clinical testing. Along this line of research, our group, at the Herlev Hospital in Copenhagen, is currently testing optimized methods of TIL manufacturing in order to apply TIL therapy in RCC.

Gastrointestinal Cancers

A high TIL density is considered a good prognostic indicator in various gastrointestinal adenocarcinomas [77]. Several studies have established the prognostic discriminatory power of immune-cell signatures (in particular cytotoxic $CD8^+$ and memory $CD45RO^+$ T cells) in colorectal cancer, including data clearly suggesting that the "immunoscore" is superior to standard staging systems [99, 100].

Thus, it is not surprising that two recent studies have demonstrated the presence of naturally occurring tumor-reactive $CD8^+$ T-cells in the tumor microenvironment of patients with gastrointestinal cancers [101, 102]. With a very high efficiency, TILs could be cultured [101, 102] and expanded to clinically relevant numbers [101].

In general, it seems that in gastrointestinal cancers the frequency of *in vitro* tumor reactive $CD8^+$ T cells is relatively low (0-3% of TILs) as compared to melanoma [101]. Therefore, it has been suggested that one of the main challenges in developing TIL therapy for gastrointestinal tumors may be the ability to selectively enrich and expand tumor reactive T-cells.

Notably, the same group has recently reported that dramatic regression of liver and lung metastases could be induced in a patient with metastatic cholangiocarcinoma after treat-

ment by *in vitro* enriched naturally occurring $CD4^+$ T cells (isolated from autologous TILs) recognizing a mutated antigen [47].

Head and Neck Cancers

A high density of lymphocyte infiltration is associated with improved outcome in head and neck squamous cell carcinoma [103, 104].

In a recent article, we characterized TILs obtained from head and neck cancer metastases. TILs were expanded with high efficiency (80% of patients, with massive expansion for up to 3,500 folds), and recognition of tumor antigens could be demonstrated in 60% of patients [105]. These data show that TIL therapy may be feasible for selected patients with head and neck squamous cell carcinoma, and pave the way for its clinical testing.

In summary, TIL therapy is now explored in cancers other than melanoma. Whether the same rules for efficacy as have been established for melanoma TIL apply to TIL treatment for other cancers, remains to be investigated. With the current technologies to enrich for tumor-reactive TIL and to define the specificity of TIL, an even more personalized approach by expanding only tumor-specific T cells for TIL infusion becomes feasible. Examples like the ability to grow tumor-specific TIL, such as from the cholangiocarcinoma patient mentioned above, demonstrate that this approach is both feasible and efficacious.

Future perspectives for TIL

Until 2010 interleukin-2 and the chemotherapeutic drug dacarbazine (DTIC) were the only Food and Drug Administration (FDA) registered treatments for metastatic melanoma, showing an objective response in a minority of treated patients without any impact on overall survival.

In 2011 the FDA approved the specific inhibitor vemurafenib of BRAF V600 for metastatic melanoma patients harboring this mutation in their malignancy [106]. Vemurafenib and later also dabrafenib [107] are highly active drugs resulting in impressive improvements in median PFS and OS in metastatic melanoma. Unfortunately, the tumor heterogeneity in metastatic disease prohibits these drugs from inducing long-term remissions, due to early tumor escape mechanisms. More recently it was demonstrated that combining BRAF inhibitors with MEK inhibitors [108-110] results in significant prolongation of PFS and probably also OS compared to BRAF inhibitors alone in BRAF V600 mutated metastatic melanoma.

In the same year, the CTLA4 checkpoint inhibitor ipilimumab was registered for the treatment of metastatic melanoma. Ipilimumab has shown ORR between 10-12% in patients

with metastatic melanoma [111-113]. Importantly, the overall survival following ipilimumab treatment reaches a plateau around 20% at 3 years, indicating that in contrast to BRAF inhibitors, ipilimumab treated patients may benefit long-term [114]. In 2014 two other drugs, pembrolizumab and nivolumab, blocking the checkpoint molecule PD-1, became available for metastatic melanoma and in 2015 nivolumab also for non-small cell lung carcinoma (NSCLC). Follow-up of patients treated with these drugs is still short, but 1-, 2-, and 3-year survival rates appear higher compared to ipilimumab. In a direct comparison pembrolizumab outcompetes ipilimumab when treating a population of naïve metastatic melanoma patients with regard to ORR and progression free survival (PFS) [112]. The combination of ipilimumab plus nivolumab has also been reported to improve ORR and PFS compared to ipilimumab [113]. With an impressive rate of complete remissions it has become clear that checkpoint inhibitors and combination of BRAF and MEK inhibitors are highly potent therapies (Table 2).

So how does TIL compare to these active treatments? When should TIL be given during the course of the cancer (Figure 3)? A direct comparison of TIL with either checkpoint inhibitors or targeted agents has not yet been done. In fact, apart from randomized controlled phase II trials comparing different TIL strategies, a RCT comparing TIL with standard of care has never been performed. In Europe, a first RCT comparing young TIL therapy to ipilimumab as first or second line treatment for metastatic melanoma has been started (ClinicalTrials.gov Identifier: NCT02278887). In the US, Lion Biotechnologies has obtained an exclusive license from the SB to develop and commercialize TIL for the treatment of metastatic melanoma and is preparing for a phase II trial in refractory patients. These strategies are directed at getting TIL therapy approved as a therapeutic option for metastatic melanoma. Compared to checkpoint inhibitors, TIL therapy has some advantages and disadvantages. TIL therapy consists of a single treatment course. Despite the toxicity that is coming from NMA, such as nausea, alopecia and bone marrow depression, and high dose bolus IL-2 with short term high fever, chills, hypotension, oliguria, hypoxia and weight gain due to fluid accumulation, practically all treated patients tolerated the treatment well and very few treatment related deaths (n = 2) have been reported (Svane, personal communication) [17]. With young TIL, especially without additional TBI, no long-term side effects have been observed, clearly showing the safety of this regimen. In up to 10% of treated, mostly refractory, patients complete remissions are induced with TIL. Especially these CR patients tend to have an excellent prognosis. Some of the deep PR patients show similar long-term survival. Prior treatment with ipilimumab does not impair subsequent treatment with TIL [25]. Whether prior treatment with PD-1 blocking agents influences subsequent TIL therapy remains to be established, but early results suggest that TIL may still be effective (Rosenberg, unpublished observation). A major disadvantage of TIL therapy is that it is laborious, patient-specific, and time-consuming, with a dropout

Table 2. Overview of response rates of other treatment modalities for metastatic melanoma

	Treatment	1-year survival rate (%)	ORR (%)	Median PFS	Median OS	CR (%)
Hodi et al, 2010	Ipilimumab 3 mg/kg	45.6	11.0	2.9 months (95% CI 2.76 – 3.02)	10.1 months (95% CI 8.0 – 13.8)	6.0
Robert et al, 2014	Dabrafenib 150 mg 2dd + trametinib 2 mg 1dd	72.0 (95% CI 67 – 77)	64.0 (95% CI 59 – 69)	11.4 months	Not yet reached	13.0
Long et al., 2014	Dabrafenib 150 mg 2dd + trametinib 2 mg 1dd	Yet unknown	67.0 (95% CI 60 – 73)	9.3 months	Not yet reached	10.0
Larking et al, 204	Vemurafenib 960 mg 2dd + cobimetinib 60 mg 1dd (for 21 days followed by 7 days rest)	Yet unknown	68.0 (95% CI 61 – 73)	9.9 months (95% CI 9.0 – NR)	Not yet reached	10.0
Weber et al., 2015	Nivolumab 3 mg/kg q2 weeks	Yet unknown	31.7 (95% CI 23.5 – 40.8)	4.7 months (95% CI 2.3 – 6.5)	Not yet reached	3.3
Robert et al., 2015	Nivolumab 3 mg/kg q2 weeks	72.9 (95% CI 65.5 – 78.9)	40.0 (95% CI 33.3 – 47.0)	5.1 months (95% CI 3.5 – 10.8)	Not yet reached	7.6
Postow et al., 2015	Ipilimumab 3 mg/kg + nivolumab 1 mg/kg q3 weeks for 4 cycles, followed by nivolumab 3 mg/kg q2 weeks	Yet unknown	BRAF WT 61.0 (95% CI 49 – 72) BRAF V600E mutated 52.0 (95% CI 31 – 73) Total 58.9%	Not yet reached	Not yet reached	22.0
Robert et al., 2015	Pembrolizumab 10 mg/kg q2 or q3 weeks	q2 weeks 74.1 q3 weeks 68.4	q2 weeks 33.7% q3 weeks 32.9%	q2 weeks 5.5 months (95% CI 3.4 – 6.9) q3 weeks 4.1 months (95% CI 2.9 – 6.9)	Not yet reached	q2 weeks 5.0 q3 weeks 6.1

Abbreviations: CI, confidence interval; CR, complete response; mg, milligrams; kg, kilogram; ORR, objective response rate; OS, overall survival; PFS, progression free survival

rate of between 20-40%. As a large part of the infused TIL appear not tumor-specific, strategies to enrich for tumor-specific TIL, for instance by selecting cells based on their phenotype (PD-1, 4-1BB) or reactivity towards tumor antigens without severely increasing culture time, should be developed. Alternatively, combining TIL with either checkpoint inhibitors or boosting TIL with neo-antigen based vaccines may increase the efficacy and outcome even further. We expect that in the coming years, these strategies are likely to be investigated and if proven safe and efficacious may replace the current standard young TIL protocol.

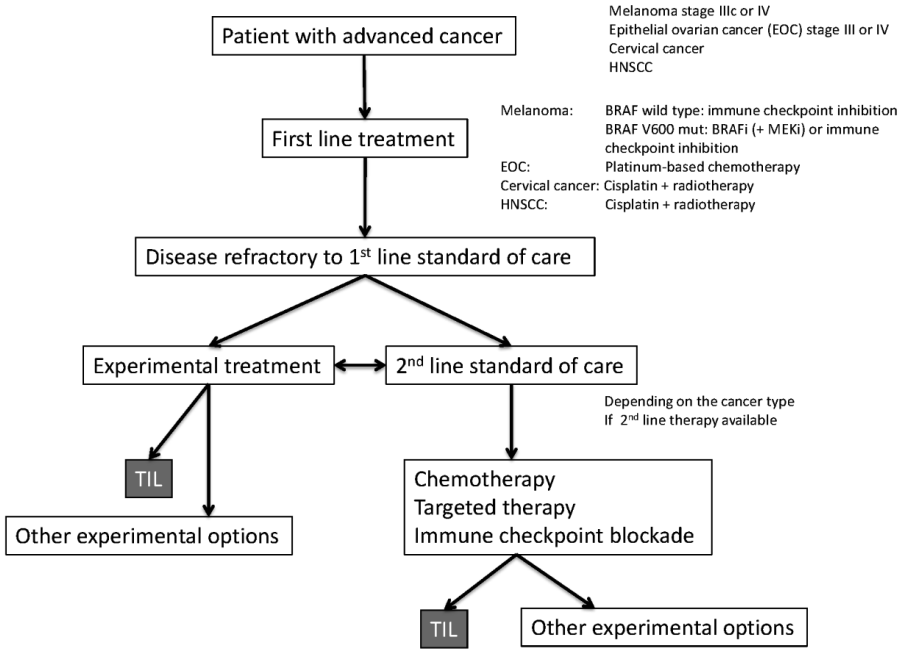

Figure 3. TIL and treatment algorithm for metastatic cancer

TIL therapy has undergone a long history of development and is still being improved to obtain the best outcome for patients. Our increasing understanding of the immunohostile tumor micro-environment, the tumor-specificity of tumor-infiltrating T cells and the development of manipulations to isolate the fittest and most tumor-reactive cells for adoptive cell therapy, will further drive the field of adoptive T cell therapy for metastatic cancers in the years ahead.

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Chapter 8

General discussion

Worldwide melanoma incidence has been rising over the past decades. For example in the Netherlands the incidence of melanoma in 1990 was around 1550 patients, while in 2015 nearly 6000 patients had been diagnosed with melanoma [1]. Approximately 10 – 14% of patients diagnosed with melanoma will eventually develop metastases [2, 3]. As previously discussed in **Chapter 1**, approved therapeutic options for metastatic melanoma until 2011 were chemotherapeutic agent dacarbazine and in some countries high dose interleukin-2 (IL-2). Several phase III trials have shown a median overall survival (OS) for dacarbazine of only 6 – 9 months [4, 5]. With the discovery of immune-checkpoints (Cytotoxic T-Lymphocyte-Associated protein 4; CTLA-4, and the Programmed cell Death(-Ligand)-1; PD-1/PD-L1 axis) and the development of monoclonal antibodies blocking these checkpoints, a great improvement in median OS has been achieved [6-10]. In the era of chemotherapy stage IV melanoma was once an almost uniformly deadly disease, with survival at 5 years ranging between 9% and 25% [11]. Now, in the era of immunotherapy, we are seeing 2-year survival rates ranging between 59% and 64%, for patients treated with anti-PD1 monotherapy or the combination of anti-PD1 plus anti CTLA-4, respectively [12]. Furthermore, a proportion of those patients will probably be cured. Nevertheless, these new drugs are costly and can induce serious, sometimes life-threatening adverse events (AEs). The discovery of biomarkers to predict upfront which patients should, and perhaps more importantly, which patients should not be treated with immunotherapeutics remains one of the goals for oncologists and researchers world-wide.

BIOMARKERS AND ADVERSE EVENTS IN IMMUNOTHERAPY

The first part of my thesis focused on the discovery of biomarkers associated with a favorable outcome on ipilimumab treatment, or OS in patients with metastatic melanoma. With the increasing numbers of immunotherapeutics developed by pharmaceutical industries we are in need for biomarkers that are predictive for response upon treatment. In my opinion there are three reasons why biomarker discovery is so important in this era: 1) biomarkers could make it possible to steer patients into their right treatment. This is especially important in patients that would not benefit from the given treatment at all. 2) All treated patients are at risk for developing serious AEs. Even though mortality rates due to treatment with immunotherapy have significantly dropped from for example 2.1% in 2010 to less than 0.2% in the years thereafter, all patients remain at risk for AEs, some of which can severely interfere with quality of life [6, 8, 10, 13]. 3) Lastly, most new treatment options against cancer are prohibitively expensive and put a serious burden on health care costs. Over the past few years many biomarkers (or combinations thereof) have been identified, but so far none have been able to provide a clear cut-off for response

to immunotherapy and targeted therapy (obviously, besides having a BRAF mutation). In **Chapter 2**, we created a model consisting of six different parameters which were gathered through routine blood parameters combined with flow cytometry. Five of these six parameters (lactate dehydrogenase; LDH, absolute monocyte counts; AMC, myeloid-derived suppressor cell frequencies; MDSCs, absolute eosinophil counts; AEC and relative lymphocyte counts; RLC) were significantly associated with OS in patients treated with ipilimumab in a multivariate analysis. Baseline values of regulatory T-cells (Tregs) were not significantly associated with OS in multivariate analysis, however the best discriminatory ability of our model was only achieved when incorporating Tregs into the model. Using this biomarker model the 2-year survival rate for patients (n = 60) with all favorable parameters (risk score = 0) was 40.8%. On the other hand, no patients (n = 38) were alive after 15 months with a risk score of > 130. We also found a statistical significant correlation between this model and best overall response rate (BORR). Patients with five favorable parameters (risk score = 0) had a BORR of 31% compared to only 3% for patients with a risk score of > 130. All parameters described in our model have already been shown to be either predictive to ipilimumab treatment or prognostic for OS in general. For example the LDH-ratio has been shown to be a strong baseline predictive biomarker and so has an increase in eosinophils after the first cycle of ipilimumab [9, 14, 15]. An interesting parameter in our model are Tregs. In our univariate analysis higher baseline frequencies of Tregs were associated with improved OS. However, this parameter was not significantly associated with OS in multivariate analysis. Nevertheless, adding Tregs to our model did provide the best discriminatory ability. Tregs are direct target cells for ipilimumab due to their constitutive CTLA-4 expression. Therefore, it seems logical that higher frequencies might render patients more susceptible to ipilimumab therapy. Elimination of Tregs due to ipilimumab works probably via antibody-dependent cell-mediated cytotoxicity (ADCC) as a result of binding of ipilimumab to CTLA-4 on the Tregs and FcγRIIIA (CD16A) present on monocytes [16]. Tarhini et al. showed that, although in a neoadjuvant setting, an increase in Tregs between baseline and week 6 was associated with an improvement in progression-free survival (PFS) [17]. On the other hand Simeone et al. showed that a decrease in Tregs, between baseline and week 12, was associated with improved survival and disease control rates [18]. Results like these show exactly how difficult it is to discover a biomarker, or combinations of biomarkers, which can perfectly distinguish responders and non-responders. Furthermore, not only blood-based parameters may have an impact on OS and/or response to ipilimumab treatment. Other known prognostic factors, such as performance status, the presence of brain metastases, or prior systemic therapies may play a crucial role on response to ipilimumab or OS in general. Probably a single biomarker, or even a combination of biomarkers, will not be able to select patients upfront that will benefit from a certain immunotherapeutic agent. It is far more likely that in the near future a biomarker-model will be established which can select patients hardly benefitting from a given treatment

at all. For example, patients in the worst possible category in our study only had a BORR of 3% to ipilimumab and no patients were alive after 15 months. In the Checkmate-067 study where patients were randomized to receive either nivolumab, ipilimumab or the combination of nivolumab + ipilimumab no patients responded to ipilimumab when their LDH was ≥ 2 x the upper limit of normal [19]. Also, in the retrospective analysis by Kelderman et al. the response rate to ipilimumab for patients with a baseline LDH of ≥ 2 x the upper limit of normal was only 7% and only 1 out of 27 patients survived longer than 12 months. For anti-PD1 therapy similar results are seen. In a recently published article Daud et al. pooled data from all 655 patients treated in the KEYNOTE-001 study. A baseline tumor burden below the median, patients with M1b disease, treatment naïve patients and patients with a normal LDH had a significantly higher BORR. Also for patients treated with the combination of nivolumab + ipilimumab a higher BORR was seen in patients with a ≥5% PD-L1 tumor expression [20, 21]. Whether these examples provided here are more prognostic in general or specifically predictive for outcome to ipilimumab treatment remains a difficult question. Recently Blank et al. proposed a framework consisting of seven parameters describing requirements for a sufficient anti-tumor immune response (the "Cancer Immunogram") [22]. We are currently analyzing the Cancer Immunogram in two cohorts of patients treated with either ipilimumab or anti-PD1 (pembrolizumab or nivolumab). Perhaps the Cancer Immunogram can help to identify melanoma patients that will, or will not, respond to immunotherapy. Future research needs to address this.

Every medicine that patients use can elicit an AE. Some of these AEs are more serious than others. This is also true for patients receiving immunotherapeutic drugs. Of these AEs some can be mild (e.g. fatigue, pruritus), some can be severe (arthralgia, vomiting) and some can be potentially life-threatening (colitis, hypophysitis, hepatitis). One of the most common AEs seen during treatment with immunotherapeutic drugs is diarrhea. The incidence of diarrhea as seen during treatment with immunotherapy is 35% for anti-CTLA-4, 20% for anti-PD1 and even 44% for the combination treatment [8, 23]. Most, if not all, studies follow the common terminology criteria for adverse events (CTCAE) to define AEs [24]. From a gastroenterologists point of view the diagnosis of colitis can only be made after inspection of the colon via endoscopy. Recently, the European Society for Medical Oncology has published an article on how to manage toxicities commonly seen during treatment with immunotherapy [25]. For diarrhea the algorithm is based on the grade of diarrhea according to CTCAE. According to these algorithms the higher the grade of diarrhea, the more aggressive therapy is indicated. In **Chapter 3** we retrospectively analyzed a cohort of 92 patients treated with immunotherapy for either metastatic non-small cell lung cancer or melanoma. Immunotherapy-related colitis (IRC) is a particular form of inflammatory bowel disease with both signs of ulcerative colitis (UC) and Crohn's disease (CD) [26]. For this reason we analyzed severity of colitis, as seen during endoscopy, according to two dif-

ferent scoring systems; the endoscopic Mayo score and the 'van der Heide' score [27, 28]. We show that there is no significant correlation between the grade of diarrhea and neither of the two scoring systems used. In the field of IBD, treatment used to be guided solely by symptoms, such as abdominal pain and diarrhea. Symptomatic treatment, however, may not improve long-term outcome or slow disease progression [29]. This is possibly due to the fact that symptoms may not accurately reflect the underlying inflammatory process characterized by ulcers. This is further highlighted in a study by Modigliani et al. in which 142 patients with active CD were included. No significant correlation could be found between clinical severity and nature, surface, or severity of endoscopic lesions. Furthermore, only 29% of patients that went in clinical remission following 1 mg/kg prednisone per day achieved endoscopic remission after 7 weeks of treatment [30]. A biomarker commonly used to assess severity of colitis is fecal calprotectin. Multiple studies have shown a strong correlation between levels of fecal calprotectin and endoscopic disease activity for colitis [31-33]. The correlation between clinical symptoms and fecal calprotectin is however much lower [33, 34]. These examples, and the data presented in **Chapter 3**, could indicate that using diarrhea as a symptom to indicate severity of colitis, and thus treatment approach, might not be optimal. We did, however, find a significant correlation between steroid-refractory colitis and the presence of ulcers and higher endoscopic scores. After failure of high-dose corticosteroids, patients are usually treated with infliximab. Treatment with infliximab in IBD has already shown to result in more clinical responses, mucosal healing, fewer hospital admissions and less surgical interventions [35, 36]. This has led to an earlier introduction of infliximab in many severe cases of IBD [37]. Future research will have to show whether an earlier introduction of infliximab in IRC will also prove to be as efficient.

BRAIN METASTASES AND LEPTOMENINGEAL METASTASES

For a subgroup of patients with metastatic melanoma, namely those with brain metastases and/or leptomeningeal metastases there is still an unmet medical need for improvement of treatment. In most large randomized phase III trials patients with untreated brain metastases were often excluded. Therefore, even in the year 2017, we simply do not know what the best treatment for these patients is. Should we withhold neurosurgery and stereotactic radiotherapy in favor of targeted therapy or immunotherapy in some of these patients? Besides local treatment and immunotherapy another possible treatment option for patients with metastatic melanoma is targeted therapy. In **Chapter 4** we analyzed a cohort of 146 patients with brain metastases from metastatic melanoma. Patients were treated with either vemurafenib, dabrafenib, or the combination of dabrafenib plus trametinib. The difference in median OS between patients treated with the combination of dabrafenib plus trametinib compared to vemurafenib was statistically significant (HR for death, 0.52;

95% CI, 0.30 – 0.89; p = 0.02). The reason that dabrafenib potentially influences OS more than vemurafenib in patients with brain metastases might be due to the fact that dabrafenib passes the blood-brain barrier (BBB) more efficiently than vemurafenib. In the European Medicines Agency (EMA) assessment report of vemurafenib it is described that concentrations of vemurafenib remained below the quantifiable limit in the brain and spinal cord [38]. Furthermore, a study by Elmquist et al. describes that the distribution of vemurafenib in the brain is severely restricted due to active efflux by P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), two major members of the efflux transporters present on the luminal side of the capillary endothelium of the BBB. Cell lines overexpressing either P-gp or BCRP significantly lowered the accumulation of vemurafenib compared to their wild-type counterpart. The difference in accumulation was abolished after a P-gp or BCRP inhibitor was added, showing that vemurafenib is a substrate for P-gp and BCRP *in vitro*. Also the area under the curve brain (AUC_{brain}) to AUC_{plasma} ratio was 0.004 in FVB (Friend leukemia virus B) wild-type mice, indicating a severely restricted brain distribution of vemurafenib [39]. On the contrary, a study performed by the same group on the distribution of dabrafenib in the central nervous system shows a higher AUC_{brain} to AUC_{plasma} ratio in the same FVB wild-type mice. The ratio for dabrafenib was 0.023, an almost 6 times higher ratio. These data indicate the greater brain penetration for dabrafenib than vemurafenib [40]. Previous data are all based on an intact BBB. However, brain metastases are known to potentially disrupt the BBB and thus making it more permeable for certain drugs [41]. For patients with metastatic melanoma without brain metastases it has already been shown that the combination of a BRAF inhibitor (BRAFi) plus a MEK inhibitor (MEKi) outperforms BRAFi monotherapy in OS and PFS [42-45]. The improved survival in patients treated with the combination regimen as compared to BRAFi monotherapy may be due to prevention of acquisition of BRAFi resistance caused by recovery of phospho-ERK signalling [46]. The recently published prospective phase 2 COMBI-MB trial also showed activity of the combination of dabrafenib plus trametinib in patients with brain metastases from melanoma. Intracranial response for asymptomatic patients without previous local brain therapy was 58% (95% CI, 46 – 69). Median PFS was 5.6 months (95% CI, 5.3 – 7.4) and median OS was 10.8 months (95% CI, 8.7 – 19.6) [47]. In 2012 Long et al. published results from the prospective BREAK-MB study [48]. In this study patients with brain metastases from melanoma were treated with dabrafenib monotherapy. In the cohort of patients that had asymptomatic, previously untreated brain metastases 39% (95% CI, 28 – 51) of patients had an intracranial response. Median PFS was 16.1 weeks (95% CI, 15.7 – 21.9) and median OS was 33.1 weeks (95% CI, 25.6 – NR). Although difficult to compare two separate studies, there appears to be a significant benefit in ORR, OS and PFS in the group of patients treated with the combination of dabrafenib plus trametinib compared to dabrafenib monotherapy. In our retrospective study ORR in the group of patients treated with dabrafenib plus trametinib was 43%, which is slightly

lower than in the study by Davies et al. However, the median intracranial PFS of 5.8 months (95% CI, 3.2 – 8.5) and the median OS of 11.2 months (95% CI, 6.8 – 15.7) are comparable. Interesting results are currently also being discovered in patients with brain metastases treated with immunotherapy. In the Checkmate 204 study patients with asymptomatic brain metastases from melanoma were treated with the combination of nivolumab plus ipilimumab. An ORR of 56% was seen and 19% of treated patients had a complete intracranial response [49]. Also in the phase II anti-PD1 Brain Collaboration study promising results are seen. In the cohort of asymptomatic patients treated with the combination of ipilimumab plus nivolumab an intracranial response rate was seen of 44% (95% CI, 24 – 65) [50]. Despite the promising results found in our study the median duration of response is rather low compared to patients treated with BRAFi +/- MEKi without brain metastases [51, 52]. Loss of the negative regulator phosphatase and tensin homolog (PTEN), resulting in an increased activation of the PI3K-AKT pathway, has been reported in brain metastases from melanoma compared to matched extracranial lesions. This has been associated with resistance to BRAF and MEK inhibitors [53, 54]. Furthermore, the increase in AKT signalling might be due to crosstalk with neighbouring cells such as astrocytes. A study by Niessner et al. has shown that metastatic melanoma cells stimulated by astrocyte-conditioned medium showed higher AKT activation than cells stimulated by fibroblast-conditioned medium [55]. Future research will have to provide us insights on the best treatment and possible combinations with local therapy (e.g. stereotactic radiosurgery, gamma knife and local surgery). Patients with leptomeningeal metastases from melanoma perhaps have the worst possible survival probability. Historical data shows a median survival of untreated patients of about two months [56, 57]. In **Chapter 5** we describe a cohort of 39 patients with leptomeningeal metastases from melanoma. A median OS of 6.9 weeks (95% CI, 0.9 – 12.8) was found for the entire cohort. Patients that received treatment with a targeted agent and/or immunotherapy had a median OS of 21.7 weeks (95% CI, 11.2 – 32.2). In the initial era of immunotherapy, treatment for leptomeningeal metastases of melanoma included intrathecal IL-2. This showed incidental responses, but also marked toxicity [58]. Ipilimumab's mechanism of action is through activation of T-cells. Activated T-cells can cross the BBB, which makes the BBB less relevant for a response within the central nervous system. This has also been shown in a study by Margolin et al. in which patients with asymptomatic intracranial and extracranial metastases from melanoma were treated with ipilimumab at a dose of 10 mg/kg once every 3 weeks. Almost similar disease control rates were seen between intracranial metastases (24%) and extracranial metastases (27%). This is further highlighted in a study in which patients with brain metastases from melanoma were treated with the adoptive transfer of T-cells, either via infusion of autologous ex-vivo expanded tumor-infiltrating lymphocytes (TIL) or autologous peripheral blood lymphocytes retrovirally transduced to express a T-cell receptor (TCR) that recognizes the melanocyte differentiation antigens gp-100 or MART-1. Seven of seven-

teen patients (41%) treated with TIL and two out of nine patients (22%) treated with TCR achieved a complete response of all brain tumor lesions [59]. Nevertheless, recently published data in a small cohort of patients (n = 16) with brain metastases that failed local therapy, were symptomatic and/or had leptomeningeal metastases treated with nivolumab (3 mg/kg q2 weeks) showed an intracranial response rate of only 6% (95% CI 0 – 30) [50]. Treating patients with ipilimumab combined with radiotherapy has shown to increase median OS in our study. We found a median OS of 47 weeks in patients treated with ipilimumab and radiotherapy. This could be partially due to the so-called abscopal effect in which increased release of tumor antigen by radiotherapy can increase antigen presentation to T-cells [60]. Future studies will have to provide us with information on the best combinatorial therapeutic regimens using immunotherapy and radiotherapy and to determine optimal timing and dosage of either treatment in patients with leptomeningeal metastases from melanoma.

ACQUIRED RESISTANCE TO BRAF INHIBITORS AND TREATMENT BEYOND PROGRESSION

Response rates to BRAFi therapy in patients with BRAF mutant metastatic melanoma are high. Response rates for vemurafenib monotherapy range between 40% and 51%, for dabrafenib monotherapy around 50% and for the combination of a BRAFi with a MEKi 63% to 70%. Nevertheless despite these high response rates median PFS is relatively short. This especially true for BRAFi monotherapy. For example, median PFS of vemurafenib is between 5.3 – 7.3 months and dabrafenib 5.1 – 8.8 months. Median PFS for the combination of a BRAFi with a MEKi is longer; 9.3 – 14.9 months [43, 44, 61-66]. Several years ago the BRAFi vemurafenib was the first and only BRAFi available on the market. In the clinic, we observed that stopping vemurafenib treatment due to disease progression would often lead to an accelerated growth of all metastases, followed by quick deterioration of the patient and death. This raised the question whether continuation of vemurafenib despite disease progression (treatment beyond progression, or TBP) could improve OS in these patients. In **Chapter 6** we retrospectively analyzed a cohort of 70 patients with metastatic melanoma treated with vemurafenib who experienced progression of disease after a prior objective response. In this cohort 35 patients stopped vemurafenib at disease progression, whilst the other 35 patients continued vemurafenib treatment despite documented progression. Median OS beyond documented progression of disease was 5.2 months (95% CI, 3.8 – 7.4) for patients that continued vemurafenib, compared to only 1.4 months (95% CI, 0.6 – 3.4) for patients that stopped vemurafenib treatment. This four month benefit in OS in this patient group is an interesting finding. Stopping vemurafenib based on progressive disease results in the growth of both resistant as non-resistant tumor cells. Treatment

beyond progression has been used with success in many different malignancies, including breast cancer, colorectal cancer and non-small cell lung cancer [67-69]. Intra-tumoral and inter-tumoral heterogeneity probably plays a crucial role in why TBP is so effective. The idea is that vemurafenib resistant tumor cells are the reason for progressive disease, while other tumor cells are still responsive to BRAFi therapy. This has been shown in the analyses of tumors progressing on BRAFi therapy in which multiple mechanisms of acquired resistance could be detected in the same tumor biopsy, but also tumors from different metastatic sites [70]. Alternatively, data from single-cell-derived resistant melanoma cells suggest that MAPKi retain some antiproliferative effect, despite signs of progressive disease [71].

CONCLUSIONS AND FUTURE PERSPECTIVES

The past few years have seen a remarkable increase in treatment options not only for patients with metastatic melanoma, but for many patients with different types of cancer. For the treatment of melanoma we are currently in an exciting era. Immunotherapy and targeted therapy have shown to increase PFS, OS and ORR in many patients compared to historical data. New combinations of checkpoint inhibitors, as monotherapy, dual therapy or even triple therapy, are currently being tested in phase 1/2 trials. Nevertheless, despite these promising results there still is a proportion of patients without a durable response upon treatment. This is especially true for patients with brain metastases and/or leptomeningeal metastases. Furthermore, while many of the new combination partners seem very promising, we need to be aware that this will require many patients to be included in future phase 3 studies, all of which may be at risk for serious adverse events. Biomarkers, such as those described in this thesis, might help us select patients in need of these new combination partners, which would allow to design smaller trials. In this thesis I have shown the importance of biomarker discovery, looked at adverse events and its management and have shown that immunotherapy and targeted therapy can have great impact in patients with brain metastases and/or leptomeningeal metastases. Future research will have to be aimed at further biomarker discovery, the discovery of new combination partners for immunotherapy and targeted therapy and better treatment options for patients with brain metastases and/or leptomeningeal metastases.

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Appendices

ENGLISH SUMMARY

This thesis focused on different aspects of melanoma treatment with immunotherapy and targeted therapy. **Chapter 1** provides an introduction into (metastatic) melanoma. Furthermore, the rationale and outline of this thesis were described. The first part of this thesis focused on biomarker discovery. Hereby making it possible to select patients upfront that should, or should not, be treated with immunotherapy. In **chapter 2** we search for biomarkers in a large retrospective multicenter study using routine blood parameters combined with flow cytometry. In a discovery cohort consisting of 105 patients from five different sites, biomarkers that were significantly correlated with overall survival were identified. These biomarkers were then validated in another cohort of 104 patients from three different sites. Five different parameters were significantly correlated with overall survival in both cohorts. Using these five parameters (lactate dehydrogenase; LDH, absolute monocyte counts; AMC, myeloid-derived suppressor cell frequencies; MDSCs, absolute eosinophil counts; AEC and relative lymphocyte counts; RLC) a model was created. However, the best discriminatory ability of this model was achieved when regulatory T-cells were also considered, despite this factor having no significant independent impact on survival according to Cox regression analysis. A nomogram-based linear predictor measure was calculated for each patient considering the relative impact of single factors according to Cox regression analyses. Patients could be divided into three groups; a risk score of 0 (low), a risk score ≤ 130 (intermediate) and a risk score > 130 (high). Using this biomarker model the 2-year survival rate for patients (n = 60) with all favorable parameters (risk score = 0) was 40.8%. On the other hand, no patients (n = 38) were alive after 15 months with a risk score of > 130. We also found a statistical significant correlation between this model and best overall response rate (the percentage of patients with a complete or partial response). Patients with all favorable parameters (risk score = 0) had a best overall response rate of 31% compared to only 3% for patients with a risk score of > 130. Another (easier) model was developed in which only the routine blood parameters were used (LDH, AMC, AEC and RLC). In this model the number of favorable parameters would be counted. Using this model the 2-year survival probability for patients (n = 141) with all favorable parameters was 43.1%, compared to 2.5% for patients (n = 109) with only 0 – 2 favorable parameters. Similarly to the first model there was also a statistical significant correlation with best overall response rate. Patients with all favorable parameters had a best overall response rate of 31% compared to 8% in the group of patients with 0-2 favorable parameters.

As already briefly discussed in chapter 1 all patients treated with immunotherapy are at risk for serious adverse events. In **chapter 3** we described a cohort of 92 patients treated with immunotherapy for either metastatic melanoma or non-small cell lung cancer. All

of these patients developed diarrhea as an adverse event for which they were treated with corticosteroids and/or underwent an endoscopy. Of all patients endoscopy images, together with pathology slides, were re-assessed. Management of immune-related diarrhea is based upon treatment algorithms that have been developed for immunotherapeutics. Immune-related diarrhea is scored according to the common terminology criteria for adverse events (CTCAE). An increase in stools per day over baseline indicates the grade of immune-related diarrhea. The treatment algorithms are based upon the grade of diarrhea according to CTCAE. According to these algorithms, the higher the grade the more aggressive therapy is indicated (e.g. symptomatic treatment for grade 1, addition of prednisone for grade 2 and the possible addition of infliximab for grade ≥ 3). We discovered that there was absolutely no statistical significant correlation between the grade of diarrhea at presentation and the severity of colitis as seen during endoscopy and quantified according to the endoscopic Mayo score. A score commonly used to assess severity of inflammatory bowel disease; ρ 0.12; $p = 0.28$. Another interesting discovery was the fact that patients in which ulcers were seen during endoscopy needed infliximab significantly more frequently than patients that did not have ulcers ($p = 0.002$).

In **chapters 4 and 5** we studied a subpopulation of patients with metastatic melanoma, namely those with brain metastases and/or leptomeningeal metastases. In **chapter 4** we retrospectively described a cohort of 146 patients with brain metastases from melanoma treated with the BRAF-inhibitors vemurafenib or dabrafenib, or with the combination of a BRAF-inhibitor with a MEK-inhibitor. In this cohort 85 patients were treated with vemurafenib, 31 with dabrafenib and 30 with the combination of dabrafenib + trametinib. We showed that median overall survival is 5.7 months for patients treated with vemurafenib, 8.8 months for patients treated with dabrafenib and 11.2 months for patients treated with the combination of dabrafenib + trametinib. The difference in median overall survival between vemurafenib and the combination of dabrafenib + trametinib was statistically significant (hazard ratio for death, 0.52; 95%, 0.30 – 0.89; $p = 0.02$). A possible explanation for this better overall survival may lie in the fact that dabrafenib has shown to penetrate the blood brain barrier to a higher extent than vemurafenib. Furthermore, the addition of the MEK inhibitor has been shown to delay BRAF-inhibitor resistance often caused by the recovery of phospho-ERK signaling. Another key aspect of **chapter 4** was to analyze the potential improvement in neurological symptoms (such as nausea, vomiting and headache) upon treatment. We showed that in 46% of symptomatic patients an improvement of neurological symptoms was seen and in 21% neurological symptoms remained stable. This is of great palliative significance. In **chapter 5** we looked into a cohort of 39 patients with leptomeningeal metastases from melanoma treated with immunotherapy or targeted therapy. Historically median overall survival has been dismal for this patient population with a median survival of only two months despite treatment with chemotherapy and/or

radiotherapy. Median overall survival for our entire population was 6.9 weeks (95% CI 0.9 – 12.8). In our cohort we showed that there is a statistically significant difference in median overall survival between treated and untreated patients (16.9 weeks versus 2.9 weeks). Especially patients treated with ipilimumab in combination with radiotherapy seemed to be doing better with a median overall survival of 47 weeks. As previously described in **chapter 2** serum LDH was also a predictive biomarker for overall survival in this cohort. Patients with a LDH higher than the upper limit of normal had a median overall survival of only 3.1 weeks, compared to 18.9 weeks for patients with a normal LDH.

Vemurafenib was the first approved BRAF-inhibitor in the treatment of patients with metastatic melanoma. Unfortunately a large percentage of patients will eventually develop progression of disease on this therapy. In **chapter 6** we described a cohort of 70 patients with metastatic melanoma treated with vemurafenib. In patients treated with chemotherapy treatment is usually stopped at progression of disease. However, in the clinic we saw that after stopping vemurafenib progression of disease would oftentimes be accelerated, quickly followed by death of the patient. We therefore retrospectively analyzed a cohort of 35 patients that stopped vemurafenib at disease progression and another cohort of 35 patients that continued vemurafenib treatment despite progression of disease. Median overall survival in the group of patients that continued vemurafenib despite progression of disease was 5.2 months (95% CI 3.8 – 7.4) versus 1.4 months (95% CI 0.6 – 3.4) for patients that stopped vemurafenib at disease progression ($p = 0.002$).

Another potent therapy against cancer is the adoptive transfer of cells, particularly of lymphocytes. In **chapter 7** we reviewed the past, present and future of patients with different kinds of cancer treated with tumor-infiltrating lymphocytes. Finally in **chapter 8** the results presented in this thesis were discussed and future perspectives are outlined.

NEDERLANDSE SAMENVATTING

Dit proefschrift vestigt de aandacht op de verschillende aspecten van de behandeling met immunotherapie en doelgerichte therapie bij patiënten met melanoom. **Hoofdstuk 1** bevat een introductie over (gemetastaseerd) melanoom. Daarnaast worden de motivering en hoofdlijnen van dit proefschrift beschreven. Het eerste deel van dit proefschrift vestigt zijn aandacht op het ontdekken van biomarkers. Met behulp van biomarkers zou het mogelijk moeten zijn om patiënten vooraf te selecteren die behandeld, of juist niet behandeld zouden moeten worden met immunotherapie. In **hoofdstuk 2** zoeken we naar biomarkers in een grote retrospectieve multicenter studie met behulp van routine bloedwaarden in combinatie met flowcytometrie. In een ontdekkingscohort bestaande uit 105 patiënten van vijf verschillende ziekenhuizen werden biomarkers geïdentificeerd die een significante correlatie hadden met totale overleving. Deze biomarkers werden daarna gevalideerd in een ander cohort bestaande uit 104 patiënten van drie verschillende ziekenhuizen. Uiteindelijk bleken vijf verschillende parameters een significante correlatie te hebben met totale overleving in beiden cohorten. Met behulp van deze vijf parameters (lactaat dehydrogenase; LDH, absolute aantal monocyten; AMC, aantallen myeloid-derived suppressor cellen; MDSCs, absolute aantal eosinofiele; AEC en relatieve aantal lymfocyten; RLC) werd een model gebouwd. Echter, bleek dit model de beste voorspellende waarde te hebben als regulatoire T-cellen werden toegevoegd aan dit model. Ondanks het feit dat deze parameter geen significante correlatie had met totale overleving. Met behulp van een nomogram werd voor iedere patiënt een score berekend aan de hand van de relatieve impact van alle losse parameters. Patiënten konden daarna worden verdeeld in drie groepen; een risico score van 0 (laag), een risico score van ≤ 130 (gemiddeld) en een risico score van > 130 (hoog). Met behulp van dit model bleek de 2-jaars overleving voor patiënten (n = 60) met alle gunstige parameters (risico score = 0) 40,8% te zijn. Aan de andere kant bleek geen enkele patiënt (n = 38) in leven na 15 maanden als zij een risico score hadden van > 130. We vonden ook een significante correlatie tussen dit model en beste respons (het percentage patiënten met een complete of partiële remissie). Patiënten met alle gunstige parameters (risico score = 0) hadden een beste respons van 31%, in tegenstelling tot slechts 3% bij patiënten met een risico score van > 130. Een simpeler model was ontwikkeld waarbij alleen routine bloedwaarden werden gebruikt (LDH, AMC, AEC en RLC). In dit model werden de gunstige parameters opgeteld. Met behulp van dit model bleek de 2-jaars overleving voor patiënten (n = 141) met alle gunstige parameters 43,1% te zijn, in tegenstelling tot 2,5% voor patiënten (n = 109) met 0 – 2 gunstige parameters. Evenals in het eerste model bleek er ook een significante correlatie te zijn met beste respons. Patiënten met alle gunstige parameters hadden een beste respons van 31% vergeleken met 8% bij de patiënten met 0 – 2 gunstige parameters.

Zoals al eerder kort in hoofdstuk 1 beschreven kunnen alle patiënten die behandeld worden met immunotherapie bijwerkingen ontwikkelen. In **hoofdstuk 3** beschrijven we een cohort van 92 patiënten die behandeld werden met immuuntherapie voor gemetastaseerd melanoom of niet-kleincellig long kanker. Deze patiënten ontwikkelden allemaal diarree als gevolg van de immuuntherapie waarvoor zij behandeld werden met hoge-dosis corticosteroïden en/of een endoscopie ondergingen. Van alle patiënten werden de endoscopie plaatjes, samen met de biopten van de pathologie opnieuw bekeken. Behandeling van immuun-gerelateerd diarree is volgens algoritmen die ontwikkeld zijn voor immuuntherapeutica. Immuun-gerelateerd diarree wordt gescoord aan de hand van de "common terminology criteria for adverse events (CTCAE)". Een toename van het aantal stoelgangen per dag geeft de graad diarree aan volgens CTCAE. Volgens deze algoritmen geldt dat des te hoger de graad diarree, des te agressiever de behandeling zou moeten zijn (bijvoorbeeld alleen symptomatische behandeling voor graad 1, toevoegen van prednison voor graad 2 en mogelijk toevoegen van infliximab voor graad ≥ 3). We ontdekten dat er geen enkele correlatie was tussen de graad diarree en de ernst van de ontsteking in de darm, zoals deze gezien werd tijdens scopie en gekwantificeerd volgens de Mayo score; ρ 0,12; p = 0,28. Dit is een score die normaal gebruikt wordt bij inflammatoire darmziekten. Een andere interessante ontdekking was het feit dat patiënten waarbij ulcera gezien werden tijdens de scopie significant vaker infliximab gegeven moest worden dan bij patiënten zonder ulcera (p = 0,002).

In **hoofstukken 4 en 5** kijken we naar een subpopulatie patiënten met gemetastaseerd melanoom, namelijk die met hersenmetastasen en/of leptomeningeale metastasen. In **hoofdstuk 4** beschrijven we een retrospectief cohort van 146 patiënten met hersenmetastasen van melanoom die behandeld werden met de BRAF-remmer vemurafenib, dabrafenib of de combinatie van een BRAF-remmer met een MEK-remmer. In dit cohort werden 85 patiënten behandeld met vemurafenib, 31 met dabrafenib en 30 met de combinatie van dabrafenib + trametinib. De gemiddelde overleving voor patiënten behandeld met vemurafenib was 5,7 maanden, 8,8 maanden voor patiënten behandeld met dabrafenib en 11,2 maanden voor patiënten behandeld met de combinatie van dabrafenib + trametinib. Het verschil in gemiddelde overleving tussen patiënten behandeld met vemurafenib en de combinatie van dabrafenib + trametinib bleek statistisch significant te zijn (hazard ratio voor overlijden, 0,52; 95%, 0,30 – 0,89; p = 0,02). Een mogelijk verklaring voor dit overlevings voordeel zou kunnen liggen in het feit dat dabrafenib de bloed hersenbarrière eenvoudiger kan passeren dan vemurafenib. Daarnaast blijkt toevoeging van een MEK-remmer er voor te zorgen dat resistentie tegen de BRAF-remmer trager optreedt. Een ander belangrijk punt van **hoofdstuk 4** was het analyseren van de mogelijke verbetering van neurologische symptomen (zoals misselijkheid, braken en hoofdpijn) na starten van de behandeling. We lieten zien dat er in 46% van de symptomatische patiënten sprake

was van een afname van neurologische symptomen en in 21% van de patiënten bleven de symptomen gelijk. Dit is van bijzondere palliatieve significantie. In **hoofdstuk 5** kijken we naar een cohort van 39 patiënten met leptomeningeale metastasen van melanoom behandeld met immunotherapie of doelgerichte therapie. De mediane overleving van deze patiëntengroep is altijd al erbarmelijk geweest met een mediane overleving van slechts twee maanden, ondanks behandeling met chemotherapie en/of radiotherapie. Mediane overleving voor ons hele cohort was 6.9 weken (95% betrouwbaarheidsinterval 0,9 – 12,8). In ons cohort lieten we zien dat er een significant verschil is in mediane overleving tussen behandelde en onbehandelde patiënten (16,9 weken versus 2,9 weken). Met name patiënten behandeld met ipilimumab in combinatie met radiotherapie lijken het beter te doen dan de rest van de patiënten met een mediane overleving van 47 weken. Zoals ook al eerder in **hoofdstuk 2** beschreven blijkt LDH een voorspellende biomarker te zijn voor mediane overleving. Patiënten met een verhoogd LDH hadden een mediane overleving van slechts 3,1 weken tegen 18,9 weken voor patiënten met een normaal LDH.

Vemurafenib was de eerste BRAF-remmer die goedgekeurd werd voor de behandeling van het gemetastaseerde melanoom. Helaas blijkt een groot deel van de behandelde patiënten uiteindelijke progressief te worden. In **hoofdstuk 6** beschrijven we een cohort van 70 patiënten met gemetastaseerd melanoom die behandeld werden met vemurafenib. In de tijd dat patiënten met chemotherapie behandeld werden, stopte men de behandeling zodra er progressie van ziekte zichtbaar was. Echter zagen wij in de kliniek dat zodra mensen met vemurafenib stopten de ziekte soms nog sneller bleek te gaan groeien, met snelle dood van de patiënt tot gevolg. Daarom hebben wij 35 patiënten onderzocht die stopten met vemurafenib bij tekenen van progressieve ziekte en 35 patiënten die doorgingen met vemurafenib ondanks progressieve ziekte. Mediane overleving van de groep patiënten die doorging met het gebruik van vemurafenib ondanks progressieve ziekte bleek 5,2 maanden te zijn (95% betrouwbaarheidsinterval 3,8 – 7,4) in vergelijking tot 1,4 maanden (95% betrouwbaarheidsinterval 0,6 – 3,4) voor patiënten die stopten met vemurafenib bij progressieve ziekte (p = 0,002).

Een andere sterke anti-kanker behandeling is adoptieve celtherapie met name van lymfocyten. In **hoofdstuk 7** beschrijven we het verleden, het heden en de toekomst van patiënten met verschillende soorten tumoren behandeld met tumorinfiltrerende lymfocyten. Uiteindelijk worden in **hoofdstuk 8** de resultaten uit dit proefschrift besproken en toekomstige perspectieven uitgelegd.

PORTFOLIO

PhD student:	drs. M.H. Geukes Foppen	M
Primary thesis advisor:	Prof. dr. J.B.A.G. Haanen	
Other thesis advisor(s):	Prof. dr. C.U. Blank	
Research programme:	40401 Experimental cancer immunology and therapy	
Title of Thesis:	Clinical aspects of immunotherapy and targeted therapy of advanced melanoma	

PhD training

	Year	Hours
Generic/disciplinary courses		
- Early Detection of Cancer	2014	16
- Basic Medical Statistics Course	2014	42
- Radiation Oncology Course	2014	42
- Postgraduate Course Advanced Immunology	2015	80
- English Writing and Presenting	2015	32
- WMO-Good Clinical Practice Training	2015	16
- Cancer Immunology and Immunotherapy	2016	56
Attended lectures, LUMC presentations, participation in meetings		
- 4th annual meeting of the society for translational oncology	2013	8
- Annual graduate students retreat	2013	24
- Dutch Tumor Immunology Meeting	2014	8
- Annual graduate students retreat	2014	24
- Multidisciplinaire WIN-O Melanoom Symposium	2014	8
- Annual graduate students retreat	2015	24
- Multidisciplinaire WIN-O Melanoom Symposium	2015	8
- 12th International Head & Neck Symposium	2015	6
- Progressing Individualised Treatment Strategies in Melanoma	2016	16

- Division seminars	2013-2017	112
- Division meeting	2013-2017	280

Congress attendance and poster or oral presentations

- Towards Personalized Immunotherapy – possible biomarkers for CTLA-4 and PD-1 inhibition (oral), Staff Evening Division of Medical Oncology, Amsterdam	2015	16
- Randomized phase III study comparing non-myeloablative lymphocyte depleting regimen of chemotherapy followed by the infusion of tumor-infiltrating lymphocytes and interleukin-2 to standard ipilimumab treatment in metastatic melanoma (poster), ASCO, Chicago	2016	40
- Clinical and radiological response of BRAF-inhibition and MEK-inhibition in patients with brain metastases from BRAF-mutated melanoma (poster), ESMO, Kopenhagen	2016	32
- Randomized phase III study comparing non-myeloablative lymphocyte depleting regimen of chemotherapy followed by the infusion of tumor-infiltrating lymphocytes and interleukin-2 to standard ipilimumab treatment in metastatic melanoma (oral), EORTC Melanoma Group Meeting, Lissabon	2016	16
- Correlation between baseline parameters and overall survival in patients with advanced melanoma treated with ipilimumab (poster), ASCO, Chicago	2017	40
- Correlation between symptoms, endoscopic features and treatment response in immunotherapy induced colitis (oral), ECCO, Amsterdam	2017	16
	Year	Hours

Lecturing, lab assistance, student supervision

- A. Scholtens (medical student), scientific internship	2014	56
- Immune-related RECIST	2016	16

TOTAL number of hours: 1034

PUBLICATIONS

MH Geukes Foppen, EA Rozeman, S van Wilpe, C Postma, P Snaebjornsson, CU Blank, JV van Thienen, ME van Leerdam, M van den Heuvel, J van Dieren, JBAG Haanen. Immune-checkpoint inhibition-related colitis: Symptoms, endoscopic features, histology and response to management. *ESMO Open. 2018 Jan 13;3*

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E Hooijberg, JB Haanen, C Blank, CR Goding, TN Schumacher, DS Peeper. A targetable dependency of MITF breaks intrinsic melanoma resistance to T cell elimination. *Submitted*

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CURRICULUM VITAE

Marnix Heimen Geukes Foppen was born in Amsterdam, the Netherlands on September 29th, 1983. He grew up with his parents and brother in Amsterdam, but lived between 1989 and 1993 in San Martino Sinzano, near Parma, Italy. He graduated from secondary school at the Nieuwe School in Amsterdam in 2002 and continued with medical training at the Academic Medical Center (University of Amsterdam). In 2007 he spent five months at the Boston Children's Hospital for a research project focusing on liver and heart iron load assessment by MRI in patients with transfusion-dependent anemias (supervision dr. Ellis J. Neufeld). After an internship at the Netherlands Cancer Institute in 2010 he graduated from medical school. After medical school he worked for one and a half years as a resident internal medicine at the Netherlands Cancer Institute, followed by one and a half years at the MC Slotervaart. In 2013 he started his PhD research at the Netherlands Cancer Institute at the department of medical oncology under daily supervision of prof. John B.A.G. Haanen and prof. Christian U. Blank. He performed research on the clinical aspects of immunotherapy and targeted therapy in patients with metastatic melanoma.

As of the 1st of September 2017, he started his specialization in internal medicine at the Rode Kruis Ziekenhuis in Beverwijk and the Academic Medical Center in Amsterdam under the supervision of dr. Hanneke S. van den Broek and prof. Suzanne E. Geerlings.