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## **Immunotherapy in advanced melanoma: crossing borders**

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CHAPTER

9

# Adoptive cell therapy in metastatic melanoma



Phase I/II study protocol to assess safety and efficacy of adoptive cell therapy with anti-PD-1 plus low-dose pegylated-interferon-alpha in patients with metastatic melanoma refractory to standard of care treatments: the ACTME trial

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## Abstract

**Introduction:** Treatment with anti-PD-1 immunotherapy does not lead to long-lasting clinical responses in approximately 60% of patients with metastatic melanoma. These refractory patients, however, can still respond to treatment with tumor infiltrating lymphocytes (TIL) and interferon-alpha (IFNa). A combination of TIL, pegylated-interferon-alpha (PEG-IFNa) and anti-PD-1 is expected to provide a safe, feasible and effective therapy for patients with metastatic melanoma, who are refractory to standard of care treatment options.

**Methods and analysis:** Patients are treated in two phases. In phase I, the safety of the combination TIL and anti-PD-1 is assessed (cohort 1) according to CTCAE 4.03 criteria. Subsequently, the safety of cotreatment with PEG-IFNa is tested in cohort 2. The efficacy will be evaluated in the second phase of the trial. Efficacy is evaluated according to RECIST 1.1 and immune-related response criteria. Clinical and immunological parameters will be evaluated for their relation with clinical responsiveness.

**Ethics and dissemination:** Ethical approval of the trial was obtained from the Central Committee on Research Involving Human Subjects in the Netherlands. The trial results will be shared with the scientific community at (inter)national conferences and by publication in a peer-reviewed journal.

**Trial registration number:** NCT03638375; Pre-results.

## Introduction

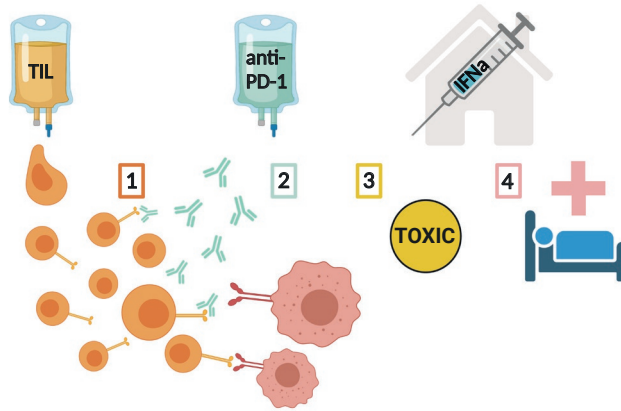
Immune checkpoint inhibition has revolutionized the treatment of metastatic melanoma in recent years. Antibodies targeting programmed cell death protein 1 (anti-PD-1) have become the new first-line standard of care immunotherapy treatment in patients with metastatic melanoma. Approximately 60% of treated patients do not have long-lasting responses<sup>(1)</sup>. The presence of sufficient numbers of activated T cells is a requirement for a durable response to anti-PD-1<sup>(2)</sup>. This condition is not always met; consequently, patients may benefit from therapies that provide these T cells, including adoptive cell therapy (ACT).

We use ACT to transfuse *ex vivo* expanded autologous tumor infiltrating lymphocytes (TIL) to the patients. The most commonly used protocol includes chemotherapy driven lymphodepletion prior to T cell infusion and concomitant administration of high-dose IL-2. This is related to serious toxicity and a long hospitalization time<sup>(3-6)</sup>. Alternatively, this conditioning and support regimen can be replaced by cotreatment with low-dose IFNa. Treatment with IFNa induces a relatively mild leukopenia, neutropenia and lymphopenia<sup>(7,8)</sup>. The combination of TIL and IFNa resulted in clinical benefit (complete response, partial response or stable disease >6 months) in 20% of patients who were progressive after prior treatment with immune checkpoint inhibition (cytotoxic T-lymphocyte-associated protein 4 antibody, anti-PD-1 or the combination of both)<sup>(7)</sup>.

We propose that the combination of ACT, with anti-PD-1 infusions and pegylated-interferon-alpha (PEG-IFNa), is a safe and effective therapy for patients with metastatic melanoma solving four of the most important aspects curtailing the efficacy and feasibility of current immunotherapies (see Figure 1).

### STRENGTHS AND LIMITATIONS OF THIS STUDY

- This is the first study to investigate the combination of a mild conditioning and supportive regimen for adoptive cell therapy and anti-PD-1.
- Study findings could be used to create a prognostic (bio)marker profile in order to select patients who will benefit most from this treatment in future protocols/studies.
- Expansion of tumor infiltrating lymphocytes is a time-consuming process, limiting the number of patients treated.



**FIGURE 1** Resolving four of the most important aspects curtailing the efficacy and feasibility of current immunotherapies: (1) providing tumor-reactive TIL; (2) alleviating immune checkpoint inhibition; (3) reducing toxicity of ACT treatment; (4) Minimalizing hospitalization and patient burden. ACT, adoptive cell therapy; IFN $\alpha$ , interferon-alpha; TIL, tumor infiltrating lymphocytes.

### Insufficient number of TIL

The magnitude of T cell infiltration in the tumor has a predictive value with respect to the natural history of primary cancers. It was shown that a greater density of tumor antigen-restricted CD8+ T cells in metastatic melanomas is associated with a better antitumor response in patients following anti-PD-1 treatment<sup>(2)</sup>. ACT delivers high numbers of activated TIL to patients. Patients with low levels of activated T cells may benefit from treatments that deliver these T cells.

### Inhibition of T cell effector function

Upregulated expression of PD-1 ligand (PD-L1) by tumor cells or tumor-infiltrating myeloid cells is one of the major mechanisms underlying immune escape. PD-L1 can bind to PD-1 on T cells and subsequently trigger inhibitory signaling downstream of the T cell receptor, blocking effector functions and reducing T cell killing capacity<sup>(9)</sup>. We showed that a substantial percentage of the infused TIL in ACT express one or more coinhibitory molecules, including PD-1. These data suggest that the full capacity of transfused T cells to control tumor cell growth may be hampered due to checkpoint inhibition<sup>(7)</sup>. Hence, the combination of TIL with anti-PD-1 may increase the tumor-reactivity of ACT.



## Toxicity of chemotherapy and high-dose IL-2

Toxicities related to the most commonly used ACT protocol<sup>(10)</sup> need to be resolved to push ACT more to the forefront of melanoma care<sup>(11,12)</sup>. These toxicities are predominantly related to the conditioning regimen, used to create lymphopenia (chemotherapy) and the high dose of IL-2 that is given to patients as a supportive regimen for the infused T cells<sup>(13-15)</sup>. The conditioning is believed to create space for the infused T cells as well as to allow their homeostatic proliferation by elimination of the cellular sinks for endogenous cytokines<sup>(3,4,16,17)</sup>.

IFNa has been shown to result in a discernible but mild and transient leucopenia<sup>(7,8,18,19)</sup> and is routinely used in allogeneic stem cell transplantation to support donor lymphocyte infusions<sup>(20)</sup>. We have observed a much lower number of adverse events when IFNa is used as conditioning and supportive regimens when compared with trials using high dose IL-2 with chemotherapy and TIL<sup>(7,8)</sup>.

## Long-term hospitalization

The previously described commonly used ACT protocol requires hospitalization for 3-4 weeks, due to the side effects of treatment with lymphodepleting chemotherapy and high-dose IL-2. As a consequence of the use of our far less toxic protocol, treatment does not require any hospitalization. Both the TIL and anti-PD-1 are given at the outpatient clinic, while PEG-IFNa subcutaneous injections are administered by patients themselves at home.

## Methods

### Study design

The ACTME study is an investigator initiated, single-center phase I/II clinical trial for patients with progressive, unresectable stage III or stage IV melanoma who are refractory to standard of care treatment options. The trial is conducted in the Leiden University Medical Center, the Netherlands.

### Eligibility and screening

Potential participants are screened by the principle investigator or one of the associate investigators, according to the eligibility criteria in box 1. Those patients found to be potentially eligible undergo baseline viral tests prior to biopsy or resection of a metastatic lesion for TIL culture.

## Study objectives

The primary objective is to evaluate the safety and toxicity of ACT with anti-PD-1, followed by evaluating the safety and toxicity of anti-PD-1, ACT plus PEG-IFN $\alpha$ , according to CTCAE 4.03 criteria.

Furthermore, the disease control rate (stable disease >6 months and partial or complete response) is evaluated according to the RECIST 1.1 criteria and immune-related response criteria (irRC). Clinical response is evaluated by overall survival (OS) and progression-free survival (PFS)<sup>(21,22)</sup>. The potential mechanisms of action of the different treatment compounds are studied and the ACT infusion product is characterized. Finally, potential correlations between the clinical response and hypothesis related immune parameters are analyzed to establish a possible prognostic biomarker profile.

### BOX 1 ELIGIBILITY CRITERIA

#### Inclusion criteria

- $\geq 18$  years old and histologically proven unresectable (or residual) regional metastatic cutaneous melanoma.
- Eastern Cooperative Oncology Group (ECOG) performance status  $\leq 1$ .
- Treated with standard treatment options (anti-PD-1, cytotoxic T-lymphocyte-associated protein 4 antibody,  $\pm$ BRAF/MEK-inhibition) and experiencing progressive disease according to RECIST 1.1.
- Within 2 weeks prior to study: hemoglobin  $\geq 6.0$  mmol/L, creatinine clearance  $\geq 60$  min/mL, aspartate transaminase and alanine aminotransferase  $\leq 5\times$  the normal upper limit, lactate dehydrogenase  $\leq 2\times$  the normal upper limit.
- Viral tests: no antibodies against human immunodeficiency viruses type 1/2, human T-lymphotropic virus, treponema pallidum, hepatitis B virus, and hepatitis C virus.

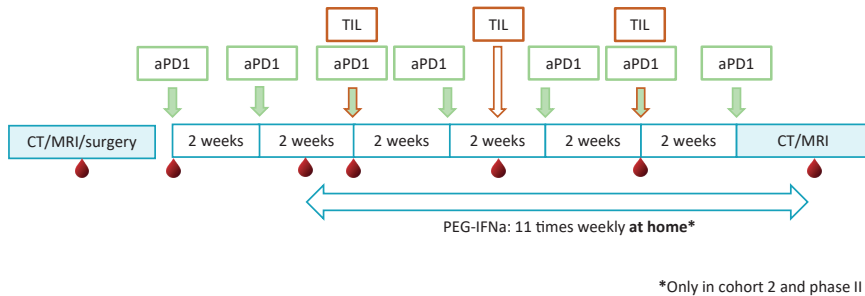
#### Exclusion criteria

- Patients with brain metastases who are neurologically unstable and/ or use dexamethasone.
- Patients with active autoimmune disease requiring immunosuppressive drugs and patients with severe autoimmune AEs following immune checkpoint inhibition therapy not related to on-target toxicity (ie, vitiligo).
- Use of systemic chronic steroid therapy ( $\geq 10$  mg/day prednisone or equivalent) or any immunosuppressive therapy within 14 days prior to start of study treatment. Topical, inhaled, nasal, ophthalmic steroids and adrenal replacement therapy are allowed.
- Other malignancy within 2 years prior to entry into the study, except for treated non-melanoma skin cancer and in situ cervical carcinoma.
- Pregnancy or breastfeeding.
- Known allergy to penicillin or streptomycin (used during the culturing of TIL).

## Study phases

The phase I part of our trial consists of two cohorts. In the first cohort, the weekly subcutaneous injections with PEG-IFNa are omitted. If the treatment with ACT and anti-PD-1 (nivolumab) is considered safe, the subcutaneous PEG-IFNa injections are added in cohort 2 (see Figure 2).

In the phase II part of the study, the patients are treated similarly to cohort 2 of the phase I part of the trial. A second cycle of PEG-IFNa, nivolumab and ACT can be added at the discretion of the treating physician, unless disease progression or complete regression of all metastases is observed during treatment evaluation at week 13. The second cycle has to be initiated within 1 month after completion of the first treatment cycle.



**FIGURE 2** Study design of ACTME trial. Blood and serum are collected at indicated time-points (red blood drop). In cohort 1, treatment with PEG-IFNa is omitted. In cohort 2 and phase II, pegylated-IFNa is added to the treatment with aPD1 and TIL. aPD1, anti-PD-1; IFNa, interferon-alpha; PEG-IFNa, pegylated-interferon-alpha; TIL, tumor infiltrating lymphocytes.

## Treatment regimen

Nivolumab is given as 2-weekly infusions at the dose of 3 mg/kg. Patients receive two infusions before the first TILs are given.

One week prior to the first TIL infusion, patients in cohort 2 and phase II start with weekly subcutaneous injections of PEG-IFNa, 1 µg/kg/week (maximum 90 µg/week). The injections are continued for 11 weeks in total (see Figure 2 and Online Supplemental Table 1).

The dose, frequency and route of administration of the TIL is similar to our previously published protocols<sup>(7,8)</sup>. We use a fixed 4-week TIL culturing period. Furthermore, based on our previous findings, we implemented a TIL dose range of  $2.5\text{-}7.5 \times 10^8$  T cells per infusion, as this was feasible in this fixed time period and because

responses to treatment were distributed among all TIL dose cohorts ( $1-2.5 \times 10^8$ ,  $2.5-5 \times 10^8$  and  $7.5-10 \times 10^8$ ) in our previous study<sup>(7)</sup>. Per treatment cycle, three TIL infusions are administered with a 3-week interval. Based on the safety data from our previous trial and data from the first patients treated in the ACTME trial, hospital admission for 24 hours following the first TIL infusion is no longer required.

### Study endpoints

Primary and secondary outcome measures are obtained through standardized clinical notes, CT scans and MRI. Furthermore, the treating physician records in the standardized clinical notes any observed treatment-related adverse events during the course of treatment and follow-up.

Scans to determine response are made at baseline and after 13 weeks.

### Follow-up

If patients have stable disease, partial response or complete response, repeat evaluations are performed every 12 weeks during the first 2 years after start of treatment. Thereafter, patients receive radiological evaluations every 4-6 months until at least 5 years after start of treatment. Patient follow-up is performed for at least 5 years or until disease progression or death.

### Outcome measures

Safety and toxicity of anti-PD-1, ACT plus PEG-IFN $\alpha$  are recorded according to the CTCAE 4.03 criteria. Toxicity grade 3 or less and serious adverse events related to treatment but not resulting in treatment termination are considered acceptable for continuation of the study.

Disease control rate is reported according to the RECIST 1.1 criteria and irRC, clinical response to treatment is defined as stabilization of disease >6 months, partial response or complete response. Survival is calculated from start of treatment to either progression (PFS), death (OS) or date of final analysis.

To study the potential underlying mechanisms of action of the different treatment compounds and to establish a possible prognostic biomarker profile, we collect blood samples at the indicated time points before, during and after treatment (see Figure 2 and Online Supplemental Table 1). Furthermore, the potential prognostic value of type of resistance (primary versus secondary) on prior immune checkpoint inhibition will be analyzed in patients treated with the combination of anti-PD-1, ACT plus PEG-IFN $\alpha$ .

### *Changes in the number and phenotype of circulating immune cells*

The measurement of absolute numbers of leukocytes, neutrophils and lymphocytes is determined by differential blood counts performed by the CKHL (central clinical and hematological laboratory) of the LUMC on the blood samples. The duration and level of leukopenia, neutropenia and lymphopenia is monitored in the subsequent blood samples.

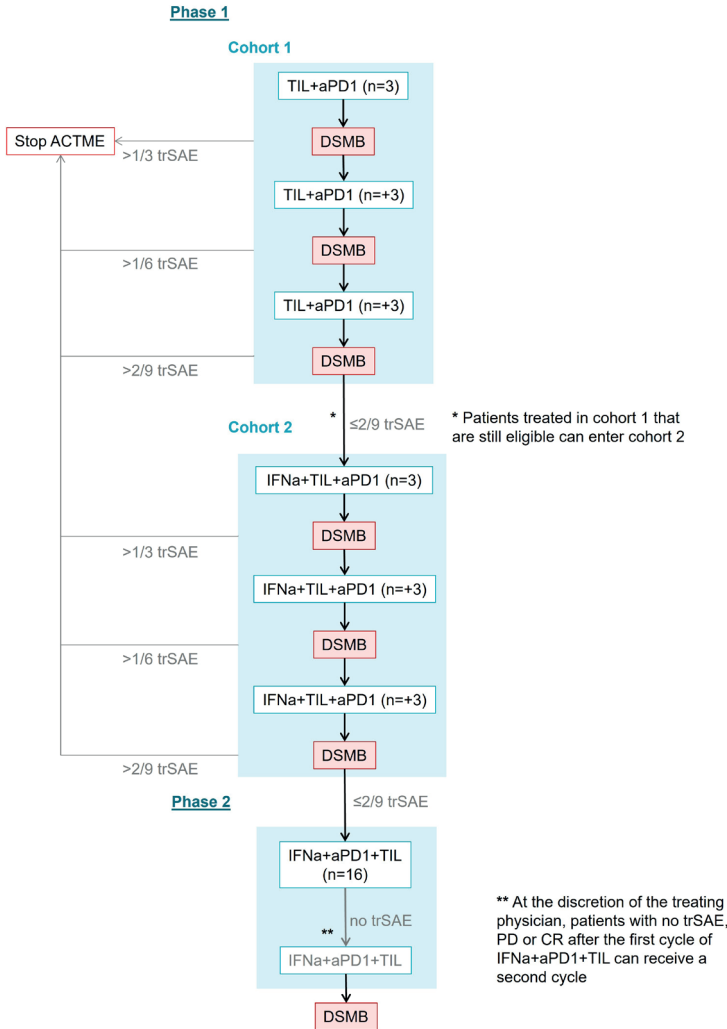
The percentage and composition of circulating immune cells may strongly affect response to immunotherapy<sup>(23)</sup>. To assess the impact of our treatments on these parameters, we use four sets of up to 11 cell surface markers to identify subsets of dendritic cells, macrophages, myeloid-derived suppressor cells, to evaluate the expression of costimulatory and coinhibitory molecules on T cells and regulatory T cells by flow cytometry, according to standard operating procedures and as was published by our group<sup>(7,24,25)</sup>.

### *Reactivity of TIL against autologous cell lines*

The reactivity of TIL to autologous tumor cells will be assessed using either a tumor cell line established from the surgery specimen or very small cryopreserved tumor fragments as stimulator cells. The frequency of activated T cells is determined by flow cytometry using the activation marker CD137 in combination with CD3, CD4, CD8, as published by us and others before<sup>(7,8,26)</sup>. The supernatants of these tumor stimulated TIL cultures are used to determine specific production of IFN- $\gamma$ , TNF $\alpha$ , IL-10, IL-5, IL-4 and IL-2 by a flow cytometer based cytokine bead array (human Th1/Th2 kit, BD) according to the manufacturer's instructions and reported earlier<sup>(7,8,25)</sup>.

### *Serum/plasma markers of persistence*

Lymphodepleting conditioning regimens are thought to support the persistence of infused T cells by increasing the serum/plasma levels of homeostatic cytokines IL-7 and IL-15<sup>(4)</sup>. The effect of PEG-IFN $\alpha$  on the serum levels of IL-7 and IL-15 collected at the indicated time points will be tested by ELISA (see Figure 2 and Online Supplemental Table 1).



**FIGURE 3** Number of patients treated per cohort and in the two study phases and data safety monitoring during ACTME trial. aPD1, anti-PD-1 treatment; DSMB; Data Safety Monitoring Board; IFNa, peginterferon-alpha2a; TIL, tumor infiltrating lymphocytes; trSAE, treatment-related serious adverse event.

**Immunohistochemistry**

A small piece of the initially removed tumor is embedded in paraffin and will be analyzed for the expression of PD-L1 and for the presence of the four-parameter signature of responsiveness, previously published by our group. These parameters include numbers of CD8+ T cells, the ratio between galectin-9+ DCs/DC-like macrophages and between M1/M2 macrophages as well as galectin-3 expression intensity<sup>(27)</sup>.

After the first treatment-cycle, surgery or a biopsy of another metastasis is performed to culture more TIL and to compare biological and immunological markers before and after treatment, both in phases I and II, when possible.

## Sample size calculation

### Phase I

The toxicity of TIL in combination with anti-PD-1, with and without PEG-IFNa, is assessed after the treatment of 9 patients in both groups (see Figure 3). The number of patients is based on a set probability of treatment related serious adverse events (trSAE) of less than 35% and was calculated using R 3.4.4 GUI statistical software for a binominal distribution. With the stopping rules as shown in Figure 3, the probability is 75% per cohort that accrual stops if the true toxicity is 35%.

A data safety monitoring board is installed to review the safety after the treatment of each three patients (see Figure 3). After completing cohort 2, an interim analysis is performed to assess the efficacy of the combination treatment. The trial will be stopped when less than two patients experience disease control after treating nine patients with PEG-IFNa TIL and anti-PD-1.

### Phase II

The main objective of the second stage of this phase I/II study is to assess efficacy of the combination of TIL, anti-PD-1 and PEG-IFNa in patients with metastatic cutaneous melanoma as determined by response rate according to RECIST 1.1.

The sample size is based on Fleming's design for single-stage phase II trials and A'Hern's adaptation of the Fleming design<sup>(28,29)</sup>. Patients eligible for this phase I/II clinical trial are refractory to the standard treatment lines. Therefore, a response rate of less than 10% ( $P_0$ ) would not be sufficiently large enough to warrant further investigation. A response rate of 30% ( $P_1$ ) or more would indicate that the combination of anti-PD-1, TIL and PEG-IFNa may be tested in a phase III setting.

Using a one-sided  $\alpha$  of 5% and 80% power ( $\beta$ ), this requires a total of 25 patients in our study ( $\alpha=0.05$ ,  $\beta=0.20$ ,  $P_0=10\%$ ,  $P_1=30\%$ ). If 6 or more out of the 25 patients have a response, then there is evidence to proceed to phase III at the end of the study. Calculated with PASS, this gives the following output showing that the actual alpha and beta are within our predefined confines:

P0	P1	Alpha	Beta	Cut-off; R+1	N	Actual alpha	Actual beta
0.1	0.3	0.05	0.2	6	25	0.033	0.193

## Data analysis plan

The primary focus of the data analysis is to determine the safety of anti-PD-1 and TIL in cohort 1. If two or less patients experience a trSAE, cohort 2 will start. In cohort 2, the primary focus is to determine the safety of anti-PD-1, TIL and PEG-IFNa. If 2 or less patients experience a trSAE, phase II starts. Only patients who completed all three TIL infusions will be included in the analyses.

In phase II, the primary focus of the data analysis is to determine the efficacy of anti-PD-1, TIL and PEG-IFNa. With a one-sided  $\alpha$  of 5% and 80% power ( $\beta$ ), 6 or more out of the 25 patients have to respond to treatment.

Descriptive statistics are used to summarize patient baseline characteristics at start of study treatment. Survival from start of treatment to progression and death is estimated according to Kaplan-Meier's method using SPSS V.25.

Paired analyses between FACS data from peripheral blood mononuclear cells (PBMC) of patients before start of anti-PD-1, at the moment of start of PEG-IFNa, at time of the first TIL infusion and after the first treatment cycle are compared using Cytosplore V.2.1.5, R V.3.4.4 and using R-package Cytofast<sup>(30)</sup>.

Furthermore, paired and independent analyses are performed on the data generated by FACS analysis on both the T cell products and the PBMC's by GraphPad Prism V.7.00 for Windows and SPSS V.25. A D'Agostino & Pearson omnibus K2 test are performed to determine whether data are normally distributed within groups. To compare paired data following a normal distribution, a paired t-test is used; when the assumption of normality is violated, a Wilcoxon signed rank test is performed. For unpaired data following a normal distribution, a unpaired t-test is used; when the assumption of normality is violated, a Mann-Whitney U test is performed.

## Ethics and dissemination

Results from our trial could increase the efficacy of ACT by overcoming four of the previously described most important aspects curtailing the efficacy and feasibility of current immunotherapies. Our outcomes will therefore be communicated to the community of oncologists treating patients with ACT during (inter)national scientific conferences, and by publication of the results in an open-access peer-reviewed international journal, the Dutch Oncology up-to-date-magazine and via the website of the Dutch Melanoma Foundation.



All patients have to give written informed consent to a member of the study team before inclusion in the ACTME study. This study is conducted according to the principles of the Declaration of Helsinki (Declaration of Helsinki, 64th WMA General Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO). The protocol is approved by the Central Committee on Research Involving Human Subjects in the Netherlands and has been prospectively registered in the U.S. National Library of Medicine (NCT03638375).

An electronic case report form is made using Castor Electronic Data Capture, where all data on patient eligibility, treatment cycles and clinical parameters will be collected by trained staff-members of the Medical Oncology Department. The clinical trial will be monitored approximately twice a year by an independent monitor.

### Patient and public involvement

Patients were involved in the design of the protocol. Patient representatives from the Dutch Melanoma Foundation will be invited to identify the key messages that need to be disseminated.

## Discussion

Current research has shown that immunotherapy with immune checkpoint inhibition is not sufficient for approximately 60% of patients. New combinations have to be implemented to overcome the mechanisms hampering current standard of care treatment options. In this phase I/II trial, we tackle the four most important aspects curtailing the efficacy and feasibility of current immunotherapies. We hypothesize that anti-PD-1 in combination with TIL and PEG-IFNa provides and maintains more activated tumor-reactive T cells, thereby improving clinical outcome while hospitalization is not required due to the acceptable toxicity profile.

We hope to complete the enrolment of the trial by mid-2023, with a 14-week follow-up first data expected by the end of 2023.

### Acknowledgements

Figure 1 was created with BioRender.com.

### Contributors

MKvdK, EMEV, EK and SHvdB designed the clinical study. G-JL performed surgery and provided the resected tumor. PMM performed QP and QC checks of the production

process and of the infusion product. ICFMR, MAJ, FMS and EK treated the patients. MKvdK, EMEV, MV, LdB and CEvdM produced the infusion product and performed all analyses. MKvdK designed the patient database. MKvdK, ICFMR, MAJ, SvdB collected data in the patient database. MKvdK wrote the original manuscript and all other authors reviewed and edited the final manuscript.

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**SUPPLEMENTAL TABLE I** Flow chart of the ACTME trial.

Week	PRE	0	2	3	4	6	7	8	10	12	13	14
History <sup>1</sup>	X			X	X		X		X			X
Physical examination <sup>2</sup>	X			X	X		X		X			X
Viral serology and HLA typing <sup>3</sup>	X											
Pregnancy test (females) <sup>4</sup>	X											
CBC differential + Blood chemistry <sup>5</sup>	X			X	X		X		X			X
Tumor metastasectomy <sup>6</sup>	X											
Nivolumab i.v.		X	X		X	X		X	X	X		
PEG-IFNa s.c. <sup>7</sup>				X	X	X	X	X	X	X	X	X
TIL infusion <sup>8</sup>					X		X		X			
Imaging studies <sup>9</sup>		X										X
Extra blood sample <sup>10</sup>	X	X		X	X		X		X			X
Plasma collection <sup>11</sup>	X	X		X	X		X		X			X
Biopsy metastasis <sup>12</sup>												X

<sup>1</sup> History includes the initial pathological confirmation of the diagnosis of malignant melanoma and during treatment the evaluation of the CTCAE 4.0 criteria. QoL assessment is performed as part of the standard care for melanoma patients.

<sup>2</sup> Complete physical examination.

<sup>3</sup> Viral serology and HLA typing: HBsAg, IgG anti-HBc, IgG anti-HCV, IgG anti-HIV1/2, HIV Ag, IgG anti-HTLV, TPHA.

<sup>4</sup> For female patients of child bearing age only.

<sup>5</sup> Blood chemistry includes: Na, K, Ca, SGOT, SGPT, LDH, gamma-GT, Alkaline Phosphatase, Bilirubin, CK, Creatinine, Blood Urea Nitrogen, glucose, serum proteins, serum albumin, C-reactive protein, free T<sub>4</sub>, TSH and cortisol. Complete Blood Count includes: white blood cell count and differentiation, red blood cell count, hemoglobin, MCV, platelet count.

<sup>6</sup> Tumor tissue is resected and used to culture TILs.

<sup>7</sup> Peginterferon-alpha-2a (PEG-IFNa); 1 µg/kg/week (max 90 µg/week) subcutaneous (s.c.) weekly, 1 week before the first TIL-infusion until week 10 (\*period of 11 weeks total).

<sup>8</sup> Tumor Infiltrating Lymphocytes (TIL) are administered.

<sup>9</sup> Tumor staging: MRI brain for judgement of cerebral metastasis, CT chest and abdomen to assess the tumor lesions in the body. Lesions must be defined according to RECIST version 1.1. The initial staging must occur as closely as possible to the first nivolumab infusion, but never more than 4 weeks apart.

<sup>10</sup> For analysis of treatment effect on immune parameters 50 ml of heparinized venous blood is obtained.

<sup>11</sup> Plasma is collected from the blood drawn for point 10 and kept stored at -20°C.

<sup>12</sup> Biopsy of a second metastasis (if feasible) is performed after the first TIL-infusion cycle for additional molecular biological and immunological tests.