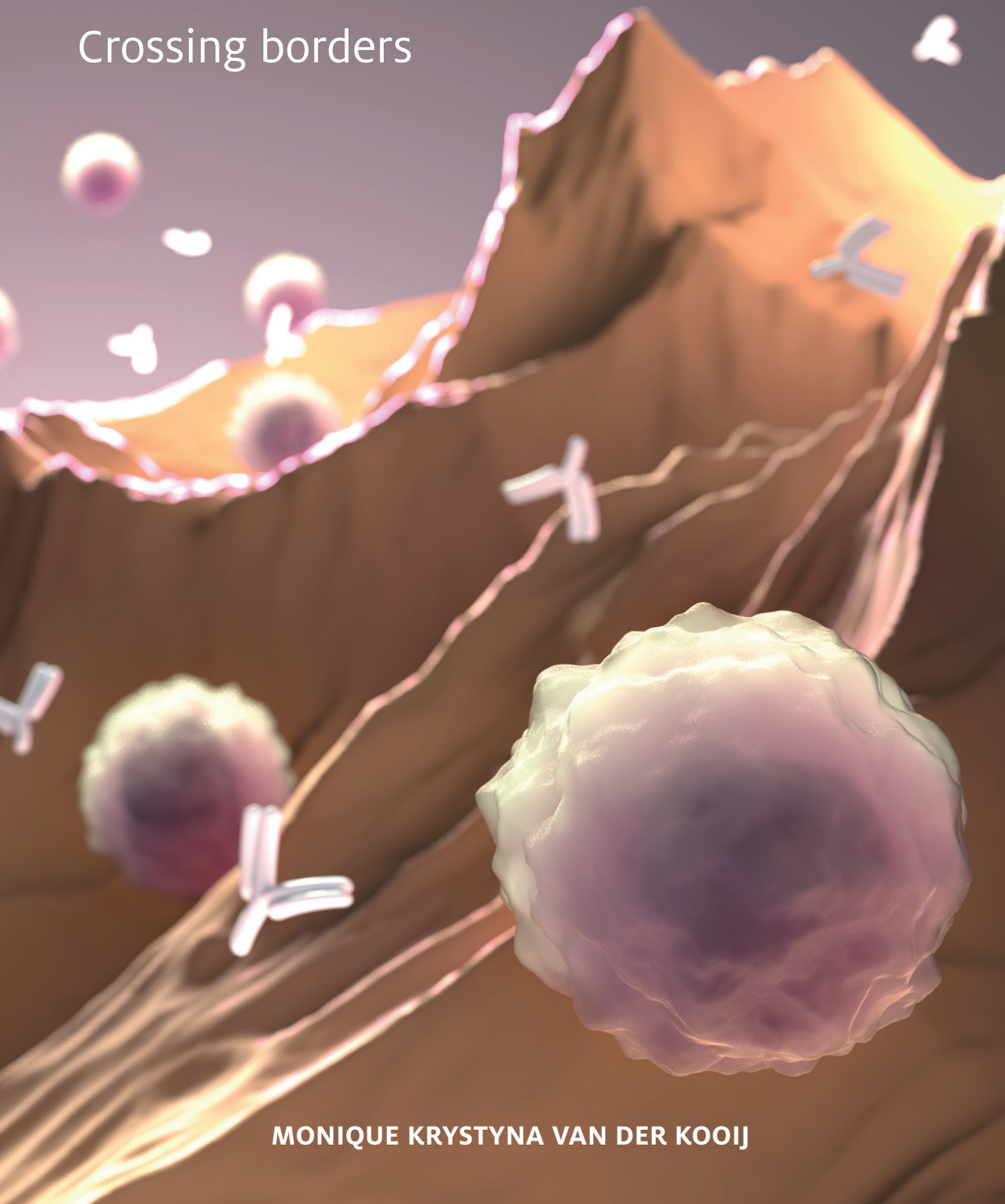
Immunotherapy in advanced melanoma: crossing borders

Kooij, M.K. van der

Immunotherapy in advanced melanoma

Crossing borders



MONIQUE KRYSTYNA VAN DER KOOIJ

Immunotherapy in advanced melanoma – crossing borders

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Voor mijn moeder

You got your passion, you got your pride

But don't you know that only fools are satisfied

Dream on, but don't imagine they'll all come true

– Billy Joel –

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CHAPTER
1

General introduction and outline

Incidence and survival

Melanoma is a deadly form of cancer that originates from melanocytes. These neural crest cells control pigmentation and are present in various parts of the human body, including the skin and uvea. Their malignant counterparts result in cutaneous melanoma (CM) and uveal melanoma (UM), respectively.

The survival rate of patients with melanoma is dependent on the stage of the disease. The staging system as defined by the American Joint Committee on Cancer (AJCC) focusses on tumor thickness, mitotic rate, and the presence of ulceration, nodal metastases, and distant metastases. Most is known about CM as the incidence is much higher when compared to UM. Further research studying the survival, treatment options, and prognostic factors in melanoma is still ongoing.

In the Netherlands, approximately 7.500 patients were diagnosed with early stage CM in 2021, and in 2020 808 patients died due to the consequences of their melanoma. Globally, the number of patients diagnosed with CM is around 325.000, with a registered mortality of nearly 57.000 patients per year[1-3]. This difference in survival between patients diagnosed in the Netherlands versus melanoma patients world-wide could be due to the more accurate registration and screening of patients in the Netherlands.

Approximately one in five patients with melanoma will develop an advanced stage of the disease (inoperable stage III and stage IV with distant metastases). When the tumor is not operable due to size or location, patients can be treated with either local treatment with radiation or talimogene laherparepvec (T-VEC) intra-tumoral injections, or systemic treatment with either chemotherapy (dacarbazine), targeted therapy (BRAF- and MEK-inhibitors), or immune checkpoint inhibitors (anti-PD-1, anti-CTLA-4, or the combination of both).

Currently, multiple clinical trials are ongoing studying the combination of abovementioned standard of care treatment options. Tumor directed treatment for advanced melanoma is evolving quickly, and is dependent on clinical characteristics of the patient, and can differ between countries. In this introduction, I will further discuss treatments and a novel combination of adoptive cell therapy and conventional immunotherapy implemented in the Netherlands.

New treatments for advanced melanoma

In recent years, multiple new treatment options have become available for patients with advanced melanoma (Figure 1). First, targeted therapy with inhibitors of the

mitogen-activated protein kinase (MAPK) pathway was introduced around 2010. This pathway is crucial in cell proliferation, cell differentiation, and cell death.

Activating mutations in the BRAF gene are present in approximately 40 to 60 percent of advanced melanomas. In 80 to 90 percent of cases, this activating mutation consists of the substitution of glutamic acid for valine at amino acid 600 (V600E mutation), in approximately 10 percent valine is replaced by lysine at the same residue (V600K mutation)[4-6]. Treatment of patients with a BRAF V600E or BRAF V600K mutation with a BRAF-inhibitor improves both overall and progression free survival[7]. Although targeted therapy is initially very effective, the tumor usually acquires resistance to these drugs within a year after start of treatment[8].

In 2011, immune checkpoint inhibition was introduced. Melanoma is one of the most immunogenic cancer types, probably due to a high mutational load[9,10]. Strong anti-cancer immunity and better clinical outcome is seen in patients with a high infiltration of T lymphocytes, presence of specific subsets of dendritic cells and dendritic cell-like macrophages, and in patients with a high M1/M2 macrophage ratio[11,12]. Cancer immunity can be inhibited by co-inhibitory signals, expressed not only by tumor cells but also by myeloid cells, both in the tumor microenvironment and the tumor draining lymph nodes[13,14].

Multiple antibodies that stimulate anti-cancer immunity by blocking co-inhibitory signals have been developed. Most well-known immune checkpoint molecules to which blocking antibodies obtained regulatory approval are Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) and Programmed Death receptor 1 (PD-1) and its ligand (PD-L1).

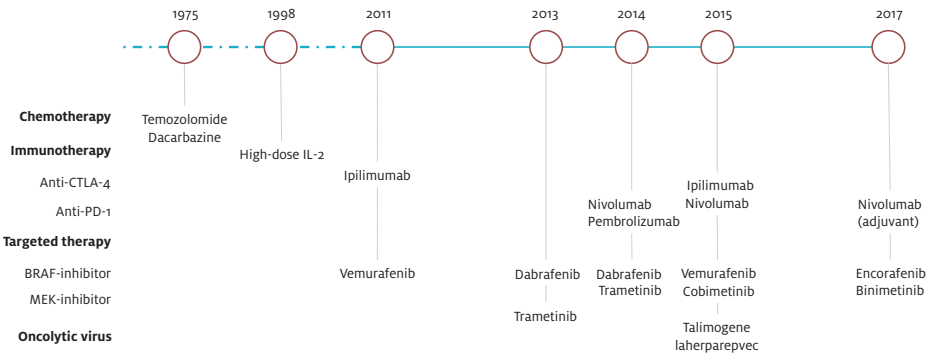

FIGURE 1 Introduction of new treatment options for patients with advanced melanoma. IL-2; interleukine-2, anti-CTLA-4; antibody against Cytotoxic T-Lymphocyte-Associated protein 4, anti-PD-1; antibody against Programmed Death receptor 1. Adapted from Van Zeijl et al. (Ned Tijdschr Geneeskd. 2018;162:D2420)

Before 2010, the median overall survival of patients with advanced melanoma was 6-9 months[15-17]. The recent 5-year follow-up data of a randomized controlled trial showed a median overall survival of 19.9 months after anti-CTLA-4, 36.9 months following anti-PD-1 and over 60 months for the group treated with the combination of anti-CTLA-4 and anti-PD-1[18]. A similar trend in prolonged annual survival rates since the introduction of these new treatments could also be observed on a nationwide scale in the Netherlands[19].

Although these results are promising, over half of the patients will not have a long-lasting response following treatment with immune checkpoint inhibition.

Furthermore, these antibodies can cause serious, and even life-threatening adverse events (AEs). In the previously mentioned trial 28%, 23% and 59% of the patients treated with anti-CTLA-4, anti-PD-1, or the combination of both experienced severe, life-threatening or disabling AEs. Most AEs are immune-related (irAE). These irAEs are thought to represent a bystander effect from activated immune cells[20,21].

Adjuvant treatment with anti-PD-1 is already approved as a standard treatment for patients with melanoma. At time of writing, trials investigating the safety and efficacy of neoadjuvant treatment with both anti-CTLA-4 and anti-PD-1 treatment are ongoing. So far, neoadjuvant treatment seems to lead to more expansion of tumor-resident T lymphocyte clones, a decrease in circulating myeloid-derived suppressor cells, and promising clinical responses. However, toxicity rates seem to be higher when compared to adjuvant therapy[22-24]. Recently, a randomized phase II trial identified a less toxic but equally effective dosing schedule for neoadjuvant ipilimumab and nivolumab in stage III melanoma[25]. An extension cohort showed that therapeutic lymph node dissection could be omitted in nearly all patients who achieved a complete or near-complete pathological response in the largest lymph node metastasis present[26].

Clinical trials and registry data

Whether a treatment gains market approval is based on data from large phase III randomized controlled trials. These large trials are considered to be the gold standard for determining the efficacy and safety of new treatments. However, these trials have strict inclusion and exclusion criteria. Overall, patients have to be in a (very) good clinical condition, with no active central nervous system metastases, and laboratory values within set parameters. The majority of the real-world advanced melanoma patients does not meet these criteria and is therefore not represented in the trials leading to market approval[27,28]. Thus, it is a matter of debate whether the results from these trials predict the response of the entire population of patients with advanced melanoma.

In July 2013 the Dutch Melanoma Treatment Registry (DMTR) was initiated. This first multipurpose nationwide registry for advanced melanoma patients registers all patients at time of diagnosis of advanced melanoma. The DMTR documents detailed information, including; tumor and patient characteristics, treatment patterns, AEs and clinical outcomes. In this thesis, I will show how databases like the DMTR make it possible to identify subsets of patients who have been excluded from large phase III trials, but can still benefit from immune checkpoint inhibition and targeted therapy[29].

Cancer immunity and new treatment options

In recent years, many clinical trials have been performed/initiated aiming to further improve the success rate of immunotherapy.

The efficacy of immunotherapy relies on a series of genetic and cellular alterations that provide the immune system of the patient with the means to generate a T cell response that recognizes and eradicates the cancer cells. This series of steps required for the final tumor eradication are part of the Cancer-Immunity Cycle, as was published by Chen and Mellman[30]. Additionally, the immune profile of an individual patient relies on an array of factors, including intrinsic tumor properties, extrinsic factors in the body, the presence of infection, and the exposure to sunlight and pharmacological agents[31].

The seven steps of the Cancer-Immunity Cycle guide our understanding of immunotherapy, treatment development over the past 50 years, and the rationale behind currently ongoing trials and new treatment combinations. For this purpose several representative treatments and trials of the many promising recent developments in the field of advanced melanoma were selected. The steps of the Cancer-Immunity Cycle are shown in Figure 2 and are described in the following text.

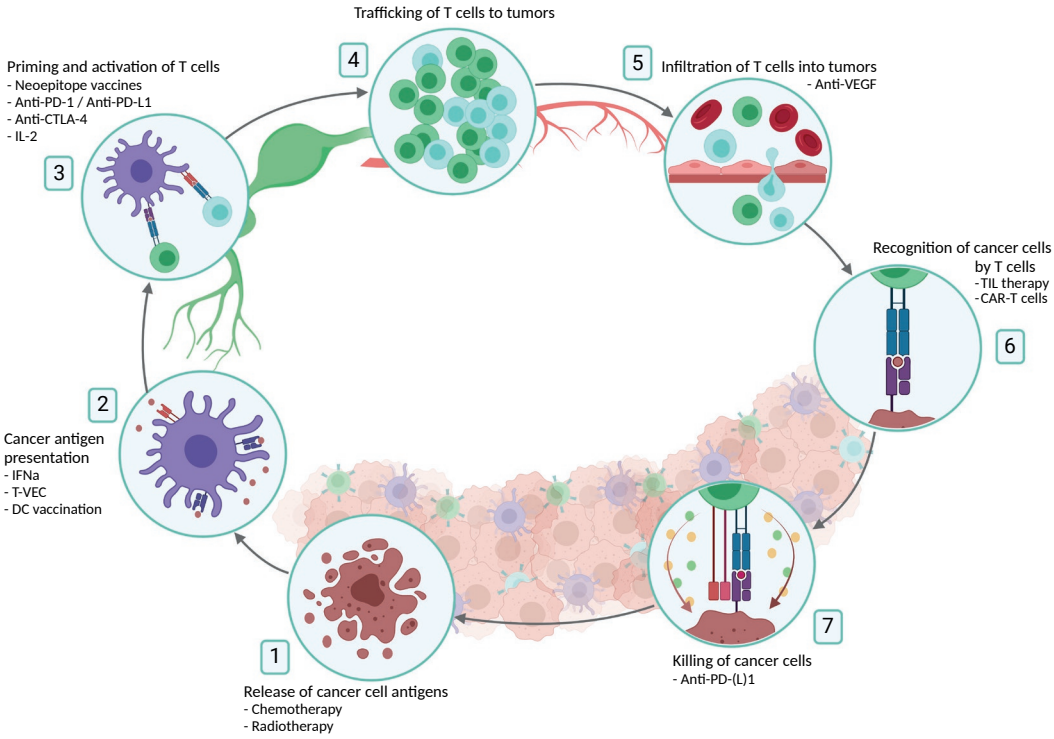

FIGURE 2 Cancer-immunity Cycle and anti-cancer treatment strategies. DC: dendritic cell, T-VEC: talimogene laherparepvec, IFNa: interferon-alpha, PD-(L)-1: programmed cell death (ligand)-1, CTLA-4: cytotoxic T-lymphocyte antigen 4, IL-2: interleukin 2, VEGF: vascular endothelial growth factor, CAR: chimeric antigen receptor, TIL: tumor infiltrating lymphocyte. This image was adapted from Chen and Mellman; Oncology Meets Immunology: The Cancer-Immunity Cycle, Immunity, Volume 39, Issue 1, 2013 (1-10), created with BioRender.com

1 Release of cancer cell antigens

In the first step tumor antigens, including neoantigens, are released after cell death, which are taken up by antigen presenting cells (APCs)[32]. These neoantigens are newly formed antigens that have not been previously recognized by the immune system. They arise from altered proteins formed as a result of mutations. As previously mentioned, melanoma has a high mutational load and therefore multiple neoantigens can be formed.

Treatment with chemotherapy and radiotherapy can lead to cell death. Currently, the only approved chemotherapy for metastatic melanoma is dacarbazine (DTIC). Since around 1970 patients have been treated with DTIC, leading to an objective response rate of approximately 20% with a median duration of 5-6 months[33]. Recently it was

shown that local treatments with radiotherapy can lead to regression of metastatic cancer at a distance. This so-called abscopal effect is mediated by activation of the immune system by the release of cancer cell antigens. Combining radiotherapy with immune checkpoint inhibitors could further enhance this effect[34].

2 Cancer antigen presentation

Once the tumor antigens are released, they have to be taken up by dendritic cells (DCs). These cells can be attracted to the tumor site by proinflammatory cytokines and factors released by dying tumor cells. These cytokines include interferon-alpha (IFNa) and tumor necrosis factor alpha (TNFa).

Treatment of melanoma with high-dose IFNa was introduced around 1985[35,36]. Studies showed that IFNa promoted tumor immunogenicity and enhanced DC attraction to the tumor, their polarization and maturation, survival and the antigen cross presentation[37,38]. IFNa also has a role in T helper 1 lymphocytes traffic to the tumor[39-41]. Three large trials by the European Cooperative Oncology Group have led to the approval of IFNa as adjuvant therapy for high-risk surgically resected melanoma (stage IIB or III)[42-44]. Clinical tumor responses in patients with metastatic melanoma were modest, with a duration of response of approximately 4 months[35].

Another way of gaining tumor antigen specific DCs in the tumor is by injecting them by DC vaccination[45]. Many clinical trials have been conducted in metastatic melanoma patients showing a moderate objective response rate of 8.5%, as reported in a meta-analysis in over 1200 melanoma patients treated with DC vaccination[46]. Interestingly, a recent study showed that following DC vaccination there is a significant increase in CD8 tumor infiltrating lymphocytes (TIL). This *de novo* inducing of a T cell inflamed tumor microenvironment was however co-occurring with the up-regulation of the T cell inhibiting signal PD-L1[47].

In 2015, vaccination with T-VEC was approved for the treatment of advanced melanoma with metastases in the skin and/or lymph nodes, based on a phase III trial with an objective response rate of 26.4%[48]. This vaccine, which is based on an oncolytic virus that is directly injected in or near the tumor, has a dual function in step 1 and step 2 of the cancer-immunity cycle. First, the genetically engineered attenuated herpes simplex virus type 1 that is injected has a lytic function and destroys tumor cells directly. This source of antigens favors local recruitment of immune cells into the tumor microenvironment. Additionally, the attenuated virus holds the gene for the human pro-inflammatory granulocyte macrophage-colony stimulating factor (GM-CSF). As the virus replicates in the tumor cells, GM-CSF is produced[49]. This cytokine promotes the recruitment and maturation of DCs and macrophages into potent APCs[50].

3 Priming and activation of T cells

Once the APCs have migrated from the tumor to a lymph node, they can present their captured antigen on MHC class I and MHC class II molecules to T cells which results in the priming and activation of an effector T cell response against these antigens. Many checkpoints and cytokines play a role in this delicate balance between suppression and overactivation of the immune system.

In 1992 the first approved immunotherapy for stage IV melanoma patients was systemic treatment with high-dose IL-2. This is a nonspecific T cell growth factor that can lead to expansion of all T cell subsets. The overall response rate was between 16-18%, with a median survival of 9.6-12 months[51,52]. Widespread use of high-dose IL-2 has mainly been hampered by its toxicity profile; capillary leak syndrome (oliguria, generalized edema, hypotension), fever, nausea, sepsis and even death.

Other more recent studies have identified several key immune checkpoints that can hamper the activation of T cells, including CTLA-4 and PD-1. CTLA-4 is an inhibitory checkpoint that is expressed on activated T cells. Once T cells recognize an antigen as non-self, a regulatory interaction occurs between the CD28 surface marker on the T cell and the molecules of the B7 family (CD80 and CD86) on the APC. This results in a stimulatory signal for the T cell. However, upon this activation CTLA-4 expression is upregulated. This can bind to CD80/CD86 with a much higher affinity when compared to CD28. If this occurs, it leads to an inhibitory signal. Blocking CTLA-4 with anti-CTLA-4 results in a more unrestrained activation of the T cell and can therefore enhance anti-tumor activity.

It was initially believed that the main interaction between PD-1 and its ligand PD-L1 occurred at the tumor site. There, recognition of the antigen presented by MHC molecules on the surface of cancer cells leads to T cell activation. Upon activation T cells produce cytokines (interferon-gamma) that induce surface expression of PD-L1 on tumor cells. This increased expression of PD-L1 inhibits the initially activated T cells. The blockade of the PD-1/PD-L1 axis results in an enhanced cytotoxic T cell response[53]. To protect DCs from cytotoxicity of activated T cells after antigen-presentation they simultaneously upregulate PD-L1. This expression on tumor infiltrating DCs plays a critical role in limiting anti-tumor immune responses[54]. More recent research revealed that tumor draining lymph nodes (TDLN) also harbor significant proportions of tumor-specific PD-1 expressing T cells, which are co-localizing with PD-L1 expressing DCs. Selectively targeting the PD-L1 in the TDLN could lead to an effective anti-tumor immune response. Therefore, it is currently believed that blockade of the PD-1/PD-L1 axis both occurs at the tumor site and the lymph nodes[13].

Instead of stimulating cancer cell death in order to increase the chances of (neo) epitopes being taken up and expressed by DCs, a recent development is treatment with patient-specific neoepitope vaccines[55]. Based on individual screening of both the tumor and healthy tissue a prediction on specific neoepitopes and their affinity can be made[56]. Multiple different vaccine formats are currently used in clinical studies, including synthetic long peptides, polyepitope DNA or polyepitope RNA[57,58]. Studies in melanoma patients with a peptide neoantigen vaccine and an intranodally administered mRNA vaccine, encoding for ten personalized neoantigens, showed that vaccination could induce a T cell response, stimulate T cell infiltration into the tumor microenvironment[59] and led to a remarkable vaccine-specific antitumor immune response[55].

Currently several clinical trials are exploring the efficacy of neoantigen vaccines in the form of peptides (NCT03639714, NCT03223103 and NCT02721043), mRNA (NCT04163094) and DNA (NCT04015700 and NCT04251117)[60] in combination with immune checkpoint inhibition.

4 Trafficking of T cells to tumors

In this step the activated T cells traffic to the tumor(s) via the bloodstream. After activation in the lymph node T cells undergo a shift in expression of surface markers and inflammation-specific receptors. By losing surface markers like CD62L and CCR7 these cells lose the ability to access lymph nodes. Instead they gain the expression of multiple homing molecules that enable them to migrate to diseased tissue. Chemokine receptors like CXCR3 bind inflammatory chemokines, including CXCL9, -10, -11 and CCL5, secreted by infected/tumor tissue[61,62].

5 Infiltration of T cells into tumor(s)

In order to be able to perform their tumor eradicating function, T cells have to migrate into the tumor microenvironment. From the bloodstream, they have to cross the endothelial lining and move through the tissue. Several proteins produced by the tumor can hamper this process. One of them is vascular endothelial growth factor (VEGF). This protein is known to drive tumor angiogenesis. Therefore, an inhibitor of VEGF was clinically studied for its proposed blood-vessel-formation control. The normalized vasculature resulted in increased tumor blood perfusion[63]. VEGF was also shown to hamper the expression of several adhesion molecules on the endothelial cells lining the tumor blood vessels[64,65]. By inhibiting VEGF there was not only better penetration of the tumor with blood vessels from which T cells could migrate into the tumor, but also the trans-endothelial cell migration and influx of these T cells was restored.

Unfortunately, no difference in overall survival was found in a randomized trial studying over 1300 patients with resected melanoma, who were treated with either adjuvant anti-VEGF or surveillance. Patients who received anti-VEGF did have a longer distant metastases free interval[66]. Interestingly, a phase I trial combining anti-CTLA-4 with anti-VEGF showed that this treatment combination was feasible and safe. Moreover, endothelial changes were present in the patients treated with this combination. Higher CD31 expression was observed in the intratumoral endothelial and interendothelial junctions. These changes were associated with extensive immune cell infiltration in the tumors, especially CD8 T cells and CD163 positive DCs[67]. At writing, the phase II follow-up trial with anti-CTLA-4 and anti-VEGF is still ongoing, as well as multiple trials combining anti-VEGF with anti-PD-1.

6 Recognition of cancer cells by T cells

Once the CD8 T cells have infiltrated the tumor, they can specifically recognize and bind to cancer cells through the interaction between its specific T cell receptor (TCR) and its cognate antigen bound to MHC class I on the surface of the cancer cell. In order to reduce the recognition by T cells, cancer cells can reduce their peptide MHC expression[68].

CD4 T cells on the other hand can exert their anticancer function in multiple ways. They can either provide signals to DCs to prime cytotoxic T lymphocytes[69], provide direct help to CD8 T cells, and in some cases they can directly recognize antigens presented by MHC class II on the surface of the cancer cell, followed by secretion of type I cytokines[70], or direct tumor killing[71].

Multiple trials have shown that both neoantigen-specific CD4 and CD8 TIL are seen in patients that respond to adoptive cell therapy (ACT)[72-75].

To increase the number of tumor infiltrating T cells, two different treatment strategies with genetically modified T cells are being implemented in the clinic. First, TCR-transgenic T cells i.e. T cells derived from peripheral blood mononuclear cells that are genetically modified by viral transduction of T cell receptors capable of recognizing specific tumor antigens[76]. Secondly, genetically modified T cells that express an artificial chimeric antigen receptor (CAR-T cell) with an antibody domain specific for recognition of a cell surface expressed tumor-specific/associated antigen and an intracellular signaling domain for activation of the T cell[77].

Since the 1980s the group of Rosenberg (NCI, USA) has been working on ACT. This process requires harvesting of TIL from the tumor, expanding them in the laboratory to large numbers and reinfusing them to the same patient. This treatment can induce

clinical responses in patients with metastatic melanoma, with the first report in 1988 describing a response rate of 50%[78,79].

In order to be successful ACT transfer requires the generation of sufficient numbers of cells with highly avid recognition of autologous tumor cells *in vitro*.

Subsequently, these activated T cells must be able to home to the tumor site in order to exert their effector function. Previous clinical trials employing the transfer of highly active antitumor T cell clones, have demonstrated that engraftment and persistence of the transferred cells required concomitant administration of high dose IL-2 to maintain cell proliferation and activation status. Rosenberg et al. reported that lymphodepletion prior to infusion of T cells can further improve the persistence and function of adoptively transferred cells. The AEs mentioned in their trial were mostly due to this high dose IL-2 that was given in combination with the ACT and included somnolence, coma, disorientation, neutropenia, thrombopenia, respiratory distress and hypotension. In later trials the group led by Rosenberg added toxic lymphodepleting chemotherapy and Total Body Irradiation (TBI) to this treatment schedule to induce a stronger lymphodepletion[80]. A more recent randomized controlled trial showed that adding TBI to lymphodepleting chemotherapy did not yield better clinical outcome. The TBI was responsible for significantly more treatment-related toxicities on top of the known toxicities from lymphodepleting chemotherapy, namely thrombotic microangiopathy, weight loss and more intesive care unit transfers and interventions[81].

The current globally used "Rosenberg-protocol" consists of; cyclophosphamide for 2 days, followed by fludarabine for 5 days. The infusion of TIL follows one day after the final dose of fludarabine. Patients subsequently receive high dose IL-2 intravenously every 8 hours up to 15 doses or until intolerance[79,82].

To date, ACT is still not part of the standard of care and is only given in clinical trials. Currently, a randomized phase III trial in the Netherlands and Denmark comparing TIL to standard anti-CTLA-4 treatment completed inclusion. The preliminary results are promising, showing that TIL treatment has a significantly longer progression free survival when compared to anti-CTLA-1 treatment. This trial is designed to open doors to lead to market approval for ACT treatment in metastatic melanoma (NCT02278887).

If ACT were to become an EMA/FDA approved treatment for metastatic melanoma, one of the important aspects curtailing the feasibility is the toxicity of the conditioning and support regimen, leading to long hospitalization and high patient burden. In the LUMC this regimen was replaced by cotreatment with low-dose IFNa.

In a phase I/II study the feasibility and safety of the adoptive transfer of tumor-reactive T cells and daily injections of IFNa in advanced-stage metastatic melanoma patients with progressive disease was tested[83]. Analysis of peripheral blood samples of the patient treated with PBMC-derived T cells with a complete clinical response revealed that circulating tumor-specific T cells persisted for at least 36 weeks after start of the infusion, sustaining the notion that T cell persistence can be achieved by daily IFNa injections instead of high dose IL-2. Additionally, treatment with IFNa induces a relatively mild leukopenia, neutropenia and lymphopenia and due to the favorable toxicity profile, this combination could be administered in the outpatient clinic.

7 Killing of cancer cells

In the final step of the cancer immunotherapy cycle, before re-entering and accelerating the whole cycle once more, T cells kill their target cancer cells. As was already described under "3. Priming and activation of T cells", one of the modes of action of anti-PD-(L)1 treatment is at the tumor site. Upon activation T cells produce cytokines that lead to the surface expression of PD-L1 in both the tumor and its micro-environment. This reactive expression of PD-L1 inhibits the initially activated T cells. The blockade of the PD-1/PD-L1 axis results in a cytotoxic T cell response[53].

The presence of high numbers of activated T cells is a requirement for a good response of PD-1 blocking therapy[84], consequently patients with low levels or absence of activated tumor-specific T cells may benefit from ACT treatment. To provide tumor-reactive TIL, alleviate immune checkpoint inhibition, reduce toxicity of ACT treatment and minimalize hospitalization and patient burden we combined ACT, with anti-PD-1 and low-dose IFNa in a new clinical trial (ACTME trial - NCT03638375).

Over the course of this PhD the clinical protocol was written, approved and the trial was initiated. The proposed mechanism of action of the treatment given in the ACTME trial is shown in Figure 3.

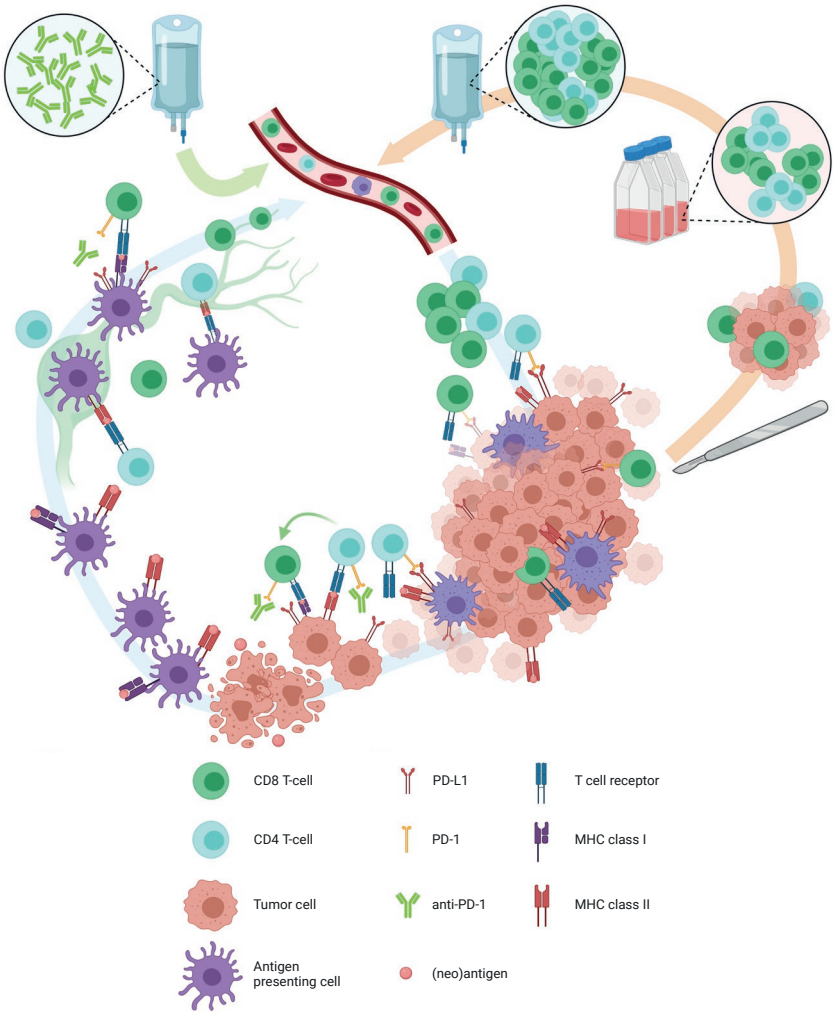

FIGURE 3 ACTME trial mode of action.

The **blue arrow** shows T cell activation. As these cells are programmed to be specific for one particular tumor antigen, they become activated after they recognize their cognate antigen in the context of an HLA molecule at the surface of an antigen presenting cell (APC). The T cell receptor (TCR) of the CD4 or CD8 T cell binds to the antigen that is presented in the MHC complex on the surface of the APC[85-89]. Upon activation a subset of helper CD4 T cells can provide critical signals to induce an adequate CD8 T cell response. Furthermore, inhibitory signals via PD-L1/PD-1 axis can inhibit the anti-tumor response of activated T cells. By using anti-PD-1 immune checkpoint inhibition it is possible to overcome this, resulting in cancer cell destruction by the patients' own T cells. Possible (neo)antigens that are released by the degrading tumor cells can be picked up by APCs to sustain the ongoing local response, or transported back to the lymphoid tissue to initiate new responses[10]. The **orange arrow** indicates the process of ACT by harvesting, culturing and reinfusing tumor-infiltrating lymphocytes (TIL) to the patient. The **green arrow** indicates the previously described systemic treatment with immune checkpoint inhibition (anti-PD-1). This figure was created with BioRender.com

Outline of the thesis

This thesis gives an overview of different treatment aspects for patients and patient subgroups with advanced melanoma and consists of three main parts. In the first part the differences between UM and CM are discussed. The second part focusses on the use of real-world data to move beyond the previously described phase III clinical trials. In this part the safety and efficacy of immune checkpoint inhibition and targeted therapy is investigated in different patient subgroups with advanced melanoma. In the third part new treatment combinations for patients with advanced melanoma are reported and discussed, including preliminary results from our ongoing trial for advanced CM patients who progressed on standard of care treatment options.

Part I

In **chapter 2** the differences in genetic alterations, metastatic routes, tumor biology, and tumor-host interactions between UM and CM is the focus. The role of the adaptive immune system differs between CM and UM. Even if immune cells succeed in infiltrating metastatic UM lesions, these cells do not seem to be activated. The described differences in CM and UM form the basis for understanding the low clinical response rate following anti-CLTA-4 (**chapter 3.1**) and anti-PD-1 (**chapter 3.2**) treatment in UM patients.

This first part of the thesis is concluded with an overview of patient characteristics, treatment options, and survival rates of advanced UM in the Netherlands in **chapter 4**. As UM is a very rare type of cancer, large trials and even data describing the current state of treatment are scarce. Using unique nationwide data, we are able to give a broad overview of all patients in the Netherlands with an advanced UM. All patients, regardless of their treatment strategy are included. The initial treatments prescribed, the corresponding overall survival, and the influence of risk factors are shown.

Part II

The second part of this thesis focusses on the use of the nationwide data from the DMTR. In **chapter 5** the treatment of advanced CM patients with and without a preexisting autoimmune disease is compared. This particular group of patients was excluded from the large trials leading to market approval of immune checkpoint inhibition, because of concerns about unleashing their underlying autoimmunity.

However, based on our findings oncologists are encouraged not to withhold immune checkpoint inhibition from patients with the more common autoimmune diseases of rheumatologic or endocrine origin. In addition, it is advised to follow-up patients

with inflammatory bowel disease closely, as severe colitis and toxicity requiring early discontinuation of treatment were higher in this group following immune checkpoint inhibition.

In **chapter 6** the focus is on adolescents and young adults (AYAs, 15-39 years of age), a group that was underrepresented in the large phase III trials with a median age of 53-62 years. We show distinct differences in primary tumor characteristics, tumor mutations, and first-line treatments initiated between AYAs and older adults. Although immune checkpoint inhibition and targeted therapy led to similar tumor responses, no AYAs experienced grade 3-4 colitis following anti-CTLA-4 treatment, while 17% of the older adults did.

In **chapter 7** potential differences in responses between male and female patients with advanced melanoma are addressed. Over the years multiple studies have been published showing conflicting results on survival and treatment response in male and female patients with (advanced) melanoma. Therefore, the question arose whether both groups can be treated with the same regimens. An overall female survival advantage of 10% was observed (**chapter 7.1**), but sex was not clearly associated with prolonged survival following immune checkpoint inhibition.

In the second part (**chapter 7.2**) the validity of an existing prediction score, claiming that female patients had a lower response to anti-PD-1 immune checkpoint inhibition when compared to male patients, was tested. This result was not validated using our extensive database, showing the importance of external validation of prediction scores.

Part III

In the final part of this thesis I discuss the results of our phase I/II clinical trial using adoptive T cell transfer in combination with low dose IFNa as treatment for stage IV cutaneous melanoma. Data on clinical results, immunological parameters and possible prognostic factors is presented in **chapter 8**. An important finding from this trial was that even patients who had previously progressed on immune checkpoint inhibition and/or targeted therapy could still respond to treatment with TIL. Furthermore, we observed that a large portion of the infused TIL expressed activation marker PD-1, which could make them more prone to inhibition via the previously described PD-1/PD-L1 axis.

These findings formed the basis for a new clinical trial that we initiated in 2018, where we combine TIL with pegylated IFNa and anti-PD-1 treatment. The rationale behind this treatment combination is described in more detail in **chapter 9.1**. The first preliminary (clinical) results from the phase I part are included in **chapter 9.2**.

General discussion

In **chapter 10** the results obtained in this thesis are discussed and implications for further research are presented.

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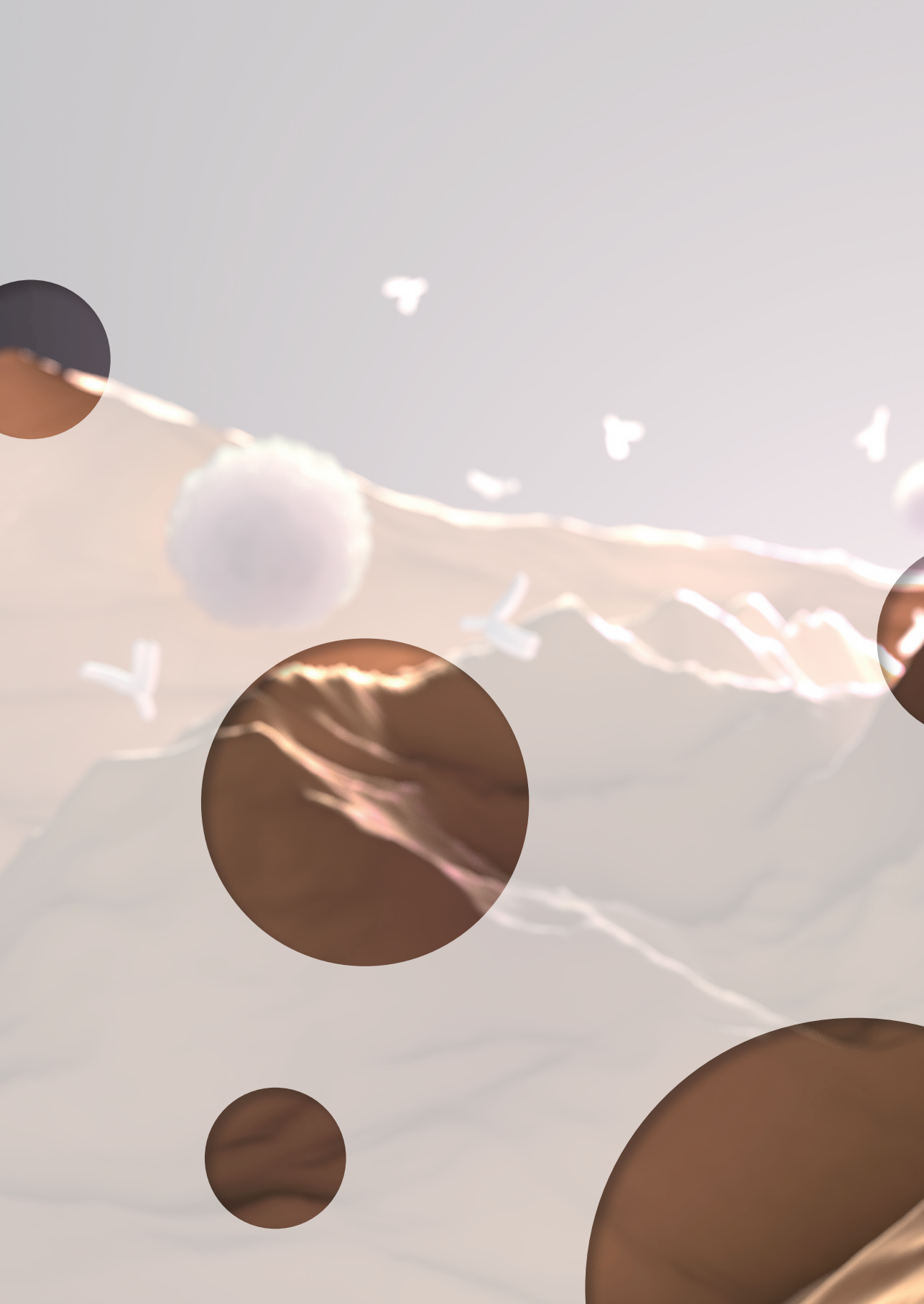

PART

I

Uveal versus cutaneous melanoma

CHAPTER
2

Uveal versus Cutaneous melanoma: Same origin, very distinct tumor types

Cancers 2019; 11, 845

Monique K. van der Kooij, Frank M. Speetjens,
Sjoerd H. van der Burg, Ellen Kapiteijn

Abstract

Here, we critically evaluated the knowledge on cutaneous melanoma (CM) and uveal melanoma (UM). Both cancer types derive from melanocytes that share the same embryonic origin and display the same cellular function. Despite their common origin, both CM and UM display extreme differences in their genetic alterations and biological behavior. We discuss the differences in genetic alterations, metastatic routes, tumor biology, and tumor-host interactions in the context of their clinical responses to targeted- and immunotherapy.

Melanocytes and Their Cellular Function

Melanocytes originate from neural crest cells and are present in various parts of the human body, including the skin, eyes, cochlea, mesencephalon, and the heart. There they are responsible for the synthesis of melanin pigments within organelles called melanosomes. In the epidermis, melanocytes transfer these melanin-containing melanosomes to neighboring keratinocytes. This ensures homogeneous pigmentation, determines skin color and protects against the harmful effects of ultraviolet radiation (UVR)[1]. In the eye, melanocytes are found in the conjunctiva and all areas of the uvea (the iris, ciliary body, and choroid). Conjunctival melanoma is distinct from uveal melanoma (UM) and shares more commonalities with cutaneous melanoma (CM)[2].

The quantity and quality of melanin pigment in the iris determines its color. In contrast to the skin, the iris color is not influenced by sun exposure. The variance in melanin expressing uveal melanocytes is associated with the occurrence of various ocular diseases, including age-related macular degeneration and uveal melanoma[3,4]. Both CM and UM arise from melanocyte transformation and represent deadly forms of cancer.

Genetic Alterations and Treatment Implications

CM and conjunctival melanoma are genetically distinct from UM. The majority of CM cases harbor mutations in proteins associated with the mitogen-activated protein kinase (MAPK) pathway. This is an important intracellular signaling pathway involved in cell growth, differentiation, and survival. Oncogenic activation of the MAPK pathway may occur via multiple mechanisms but most commonly is driven by a constitutively activated mutated *BRAF* kinase. *BRAF* kinase mutations are present in 40-60% of the CM patients, 97% of which is located in codon 600.

BRAF-mutated melanoma tends to exhibit distinctive clinical features and is characterized by a more aggressive biological behavior than *BRAF* wild-type (WT) melanoma. *BRAF*-mutated melanoma may be associated with shorter overall survival and adverse prognostic factors, but this is still under investigation[5-8]. The second most common MAPK pathway aberration in CM is mutated *NRAS*, occurring in 15-30% of patients (Figure 1)[9-12]. Melanoma with mutations in the stem cell factor receptor tyrosine kinase gene (*KIT*) represents a relatively rare subset, seen in roughly 20% of mucosal, acral, and chronically sun-damaged skin[13].

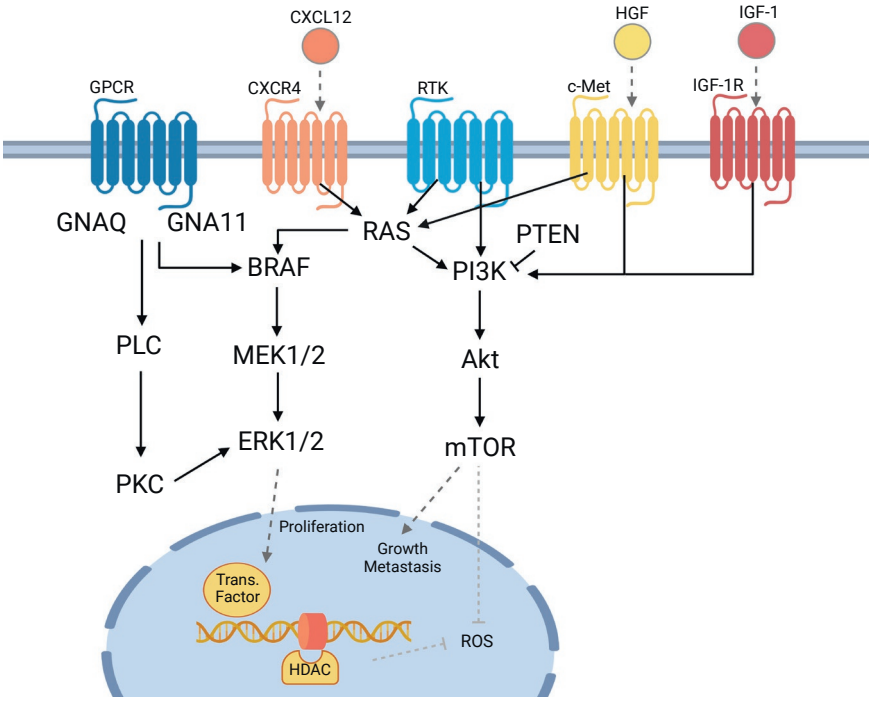

FIGURE 1 Signaling pathways and receptors involved in uveal melanoma (UM) and cutaneous melanoma (CM). Three main signaling pathways affected in UM and/or CM patients are depicted. G protein-coupled receptor (GPCR) with its Guanine nucleotide-binding proteins: the first is the Guanine nucleotide binding protein (*GNAQ*) and subunit alpha-11 (*GNA11*), which downstream activate Phospholipase C (PLC) and Protein Kinase C (PKC). The second is the mitogen-activated protein kinase (MAPK) signaling pathway, consisting of *BRAF-MEK1/2-ERK1/2*. Finally, there is the *PI3K/Akt/mTOR* pathway, which can be influenced by both RAS (from the MAPK signaling pathway) and phosphatase and tensin homolog (PTEN). The previously described chemokine receptors and their influence on the signaling pathways are added: C-X-C chemokine receptor 4 (CXCR4), with its C-X-C Motif Chemokine Ligand 12 (CXCL12), tyrosine-protein kinase Met (c-Met) and its ligand Hepatocyte Growth Factor (HGF), and Insulin-like Growth Factor-1 Receptor (IGF-1R), with Insulin-like Growth Factor-1 (IGF-1). In the nucleus, the ERK1/2 stimulates transcription factors, while both histone deacetylase (HDAC) and mechanistic target of rapamycin (mTOR) inhibit the formation of Reactive Oxygen Species (ROS). Figure was created with BioRender.com.

The discovery that many CM are caused by a mutation in *BRAF* kinase has led to the development of selective inhibitors of the *BRAF* V600-mutated kinase (vemurafenib, dabrafenib, and encorafenib) and inhibitors of the downstream *MEK* kinase (trametinib, cobimetinib, and binimetinib). *BRAF* inhibition results in high response rates in patients with a *BRAF* V600E or V600K mutation; however, most patients ultimately develop acquired resistance. The combination of BRAF and MEK inhibitors is more effective in forestalling the development of acquired resistance when compared to BRAF monotherapy[14]. Five large phase III randomized controlled trials

reported a median progression free survival for the combination treatment with BRAF and MEK inhibition of 9.3-11.4 months whereas this was 5.8-8.8 months for treatment with a BRAF inhibitor and placebo[15-19]. The treatment with KIT inhibitors improved the overall survival of patients with *KIT*-mutated gastro-intestinal stromal tumors. Following this success, multiple trials have shown that patients with metastatic melanoma harboring a *KIT* mutation were responsive to therapy with KIT inhibitors imatinib, sunitinib, dasatinib, and nilotinib[13]. The response rates in patients with metastatic melanoma are around 20-25%, when all *KIT* genetic lesions are considered, and reach 35-50% in melanomas with a KIT mutation in exon 11 or 13[20-24].

Mutations in *BRAF* V600E occur in 29-50% and mutations in *NRAS* occur in up to 18% of the patients with a conjunctival melanoma. *KIT* mutations have only been reported in one conjunctival tumor[25,26]. As it is a rare form of ocular melanoma, clinical data after BRAF inhibition is scarce. Two case reports show mixed results[27,28]. However, the genetic similarities suggest that treatment regimens used for metastatic CM should be further investigated in metastatic conjunctival melanoma. In UM, the most commonly mutated genes are *GNA11*, *GNAQ*, *BAP1*, *EIF1AX*, and *SF3B1*.

More than 90% of the UM exhibit a mutation in *GNA11* or *GNAQ*, which activate signaling between G-protein-coupled receptors and downstream effectors as well as upregulate signaling of the MAPK pathway (Figure 1)[29,30]. These mutations occur mutually exclusive in the majority of uveal melanomas, and are considered an early event in the development of UM. Mutations in *GNAQ* and *GNA11* are not associated with a worse prognosis or with the development of metastatic disease[31-34].

However, primary UM can be stratified into four distinct, clinically relevant molecular subtypes with a significant difference in metastatic rate and prognosis[30]. Class 1A and 1B tumors retain a differentiated melanocyte phenotype, with a disomy of chromosome 3. They are further distinguished by alterations in either *EIF1AX* or *SF3B1*, respectively, with 1A having a lower metastatic rate when compared to 1B. Class 2 UM is associated with a high metastatic risk and is characterized by a monosomy of chromosome 3, followed by aberrancies in *BAP1* expression and global DNA methylation. A further subdivision can be made into class 2A and 2B based on chromosome 8q copy number alterations, RNA expression, and cellular pathway activity profiles[35]. With Class 2B having a higher metastatic rate when compared to Class 2A[35-37].

As most UM are characterized by mutations in *GNAQ* or *GNA11*, therapies that target downstream effectors of these pathways such as *MEK*, *Akt*, and protein kinase C (PKC) are being investigated. Unfortunately, the results have been disappointing with response rates generally less than 10%[38,39]. A promising new target in UM could be epigenetic dysregulation. As previously mentioned, somatic mutations in the

tumor suppressor gene *BAP1* are correlated with metastatic behavior[40]. The loss of *BAP1* seems to sensitize UM cell lines to treatment with histone deacetylase (HDAC) inhibitors. HDAC induces a G1 cell cycle arrest with an increased cyclin D1, impaired cell proliferation, growth reduction, and induction of apoptosis in UM both *in vivo* and *in vitro*[41-43].

Treatment with HDAC inhibitors might prove to be beneficial for both UM and CM, as the balance between histone acetylation and deacetylation is altered in multiple cancer types. This balance defines the level of acetylation of histone and therefore plays a critical role in the regulation of gene expression[44]. While histone acetyltransferases (HAT) mediated acetylation is associated with gene transcription, HDAC-mediated histone deacetylation is associated with gene silencing. Inhibition of HDAC was shown to block tumor cell proliferation and differentiation. Currently, there are four HDAC inhibitors approved by the FDA for treatment of cancer; vorinostat, romidepsin, belinostat for T cell lymphoma, and panobinostat for multiple myeloma[45]. Several trials are studying the effect of HDAC inhibition in patients with UM or CM. Furthermore, there is pre-clinical evidence that combining HDAC inhibitors with conventional immunotherapies, targeted therapies, or cyclin-dependent kinase (CDK) inhibitors might work synergistically[46-48].

Biological Parameters Underlying Metastasis

Cutaneous and ocular melanomas have distinctly different clinical courses. For both CM and UM, the development of metastatic disease is an important determinant of the clinical course and survival.

CM tends to spread via the lymphatic system, mostly to the lungs, brain, lymph nodes, and soft tissue, with 14-20% of patients developing liver metastases[49]. Because there are no lymphatics in the uveal tract, ocular melanoma spreads hematogenously, resulting in the liver as the predominant metastatic site (89% of cases)[50].

The striking liver tropism of UM metastasis is currently not fully understood. In 1889, Paget introduced the concept of "seed and soil", which proposed that the spread of tumor cells is governed by interaction and cooperation between the tumor and the host organ[51]. More recent studies have provided a better understanding of the process of metastatic spread of multiple cancer types, including melanoma[52]. One of these studies showed that some tumors succeed in creating a premetastatic niche in the liver. They manipulate the microenvironment of different organs to render them more permissive to metastatic outgrowth before the cancer cells actually enter the organ. It was shown that integrin expression profiles of circulating plasma exosomes isolated from amongst other CM and UM could be used as a prognostic factor to predict sites of future metastasis[53].

Furthermore, a wide variety of tumors express chemokine receptors corresponding with the expression of their respective ligands in the organs bearing the highest frequency of metastases. Chemokine receptors might also influence the overall survival in patients, and may present as potential targets for treatment.

(1) CCR4-CCL17/CCL22 axis: in CM, it was shown that CCR4 overexpression might enhance the tumor's potential to metastasize to the brain[54].
In UM, no correlation between this axis and metastatic pattern has thus far been described[55].

(2) CCR7-CCL19 axis: in CM, the CCR7-CCL19/CCL21 axis is associated with regional lymph node metastases[56,57].
In UM, the expression of CCR7 seemed to be correlated with the development of liver metastases.
Both in CM and UM, this axis has been correlated with a worse patient outcome[58,59].

(3) CCR10-CCL27 axis: in a CM preclinical model, it was shown that CCR10 might play an important role in sustaining tumor viability, in protecting cells from the immune response, and in the dissemination to the draining lymph node. High expression of CCR10 was associated with a worse overall survival[57,60,61].
In UM, no correlation was found between the presence of CCR10 and/or CCL27 and the formation of liver metastases[62].

(4) CXCR3-CXCL9/CXCL10 axis: stimulation of this axis has been described to be have both pro-tumor and anti-tumor effects. This may be due to the different effects of the ligands on CXCR3. CXCL9 predominantly mediates lymphocytic infiltration and suppresses tumor growth. The induction of both CXCL9 and CCL10 expression was also seen in CM patients that responded well to interleukin 12 immunotherapy[63]. Furthermore, stage III CM patients with CXCL10 expressing CD8 T cells had a better overall survival. Conversely, CXCR3, the receptor for both CXCL9 and CXCL10, is associated with thicker primary tumors, the absence of lymphocytic infiltration, and the presence of distant metastases. It has been shown that the anti-tumor effect of this axis is induced by paracrine activation by immune cells, while the pro-tumor effect is caused by autocrine signaling mainly through the CXCR3A ligand in cancer cells[64]. The selective targeting of CXCR3A was therefore suggested to be an effective treatment option in metastatic disease.
In UM, it has been shown that CXCL10 is upregulated in a T cell-rich environment. Recently, it was shown that in UM, mainly activated macrophages express this lymphocyte-homing chemokine CXCL10. Furthermore, CXCL10 expression may serve as an independent risk factor, inversely correlated with survival[36].

(5) CXCR4/CXC7-CXCL12 axis: in CM, high CXCR4 expression is associated with the presence of tumor ulceration, thicker lesions, as well as shorter disease-free survival, time to metastasis, and overall survival. Furthermore, its expression is associated with the development of liver and lung metastases[65,66].
The expression of CXCR4 on UM cells and the presence of CXCL12 in the liver offers an explanation for the selective colonization of the liver by UM. Interactions between CXCR4 and CXCL12 stimulate tumor cell migration and invasion of basement membrane preparation by increasing the formation of cell adhesion molecules like matrix metalloproteinases[59,67]. CXCL12 also stimulates proliferation and survival of CXCR4 positive tumor cells[68-70]. Furthermore, chemotaxis of uveal melanoma cells could be inhibited by anti-CXCR4[59].

(6) c-Met, a receptor for hepatocyte growth factor (HGF): In CM overexpression of c-Met is associated with tumor growth and metastasis. Inhibition of HGF induced c-Met proliferation reduced melanoma cell line migration and invasion *in vitro*[71].
In UM c-Met also promotes tumor invasion and stimulates tumor growth[72]. The expression of c-Met in primary UM increases the risk of subsequent liver metastasis[73]. Cabozantinib is a tyrosine kinase inhibitor that targets the MET, AXL, and vascular endothelial growth factor (VEGF) receptors. In CM cells it inhibits HGF-induced migration and invasion[74], while in an UM xenograft model, it was shown to reduce hepatic metastasis[75]. A recent phase II randomized discontinuation trial in which the MET/VEGF receptor inhibitor cabozantinib was tested, revealed clinical activity in both metastatic CM and UM patients[76].

(7) Insulin-like growth factor-1 (IGF-1) plays an important role in tissue growth, and increases the risk for the development of many tumor types, including CM[77]. Both in CM and UM the serum IGF-1 level functioned as a potential predictive biomarker for metastatic disease. Strikingly, whereas metastatic UM patients displayed lower IGF-1 serum levels when compared to healthy controls, the IGF-1 serum levels were higher in metastatic CM patients[78,79]. In UM, a high expression of the IGF-1 receptor (IGF-1R) was found in hepatic metastasis and related to death due to metastatic disease[80-82]. The IGF/IGF-1R axis has been a target for new treatment combinations in both CM and UM. In CM, IGF-targeting agents have been used in combination with other treatment modalities, as it plays a role in both primary and acquired treatment resistance[83]. Preclinical research shows promising results when IGF-1R inhibition is combined either with PI3K inhibition, Stat3 blocking, or chemotherapy (temozolomide)[84-86]. In metastatic UM, a trial treating patients with an anti-IGF-1R antibody (IMC-A12, cixutumumab), was conducted. However, the final results have not yet been published (NCT01413191).
Hypoxia-inducible factor (HIF) plays a key role in tumorigenesis and metastasis in multiple types of cancer[87]. It plays an important role in the development of

CM from melanocytes. Even at normal oxygen levels, HIF activity is increased in melanoma, thereby accelerating the invasion of tumor cells into adjacent tissues and providing sufficient blood supply[88,89]. Recently, FBXO22 was introduced as a possible new treatment option for CM as it is supposed to regulate the expression of HIF[88].

In UM it was shown that relative activity of hypoxia differentiated the subgroups, irrespective of chromosome 3 status[35]. Both the previously mentioned c-Met and CXCR4 are important surface mediators of hypoxia-induced migration, invasion, and metastasis[90,91]. In addition, elevated mRNA expression of both MET and CXCR4 was found in patients with a poor prognosis and the expression levels of CXCR4, c-Met, and HIF-1 were higher in the primary tumor of patients with a subsequent metastasis. Furthermore, in cell cultures hypoxia can induce c-Met and CXCR4 expression, while these effects were inhibited by a HIF pathway inhibitor (arylsulfonamide 64B) both *in vitro* and in an *in vivo* orthotopic mouse model. *In vivo* treatment resulted in inhibition of primary UM growth, less liver metastasis formation, and a better survival[92].

The Impact of the Immune System

1.1 Primary Tumor

The distribution of immune cells varies between different tumor types. In CM, the role of the adaptive immune response in controlling tumor progression has gained a lot of attention over the past decades. In primary CM the presence of CD3+CD8+ lymphocytes, specifically activated (HLA-DR expressing) CD8+ T cells, in both the tumor and the stroma was correlated with disease-specific survival[93].

Multiple studies have investigated the role of immunosuppressive regulatory T cells (Treg) in primary CM, with conflicting results. This might be due to differences in phenotypic markers used or technical differences in staining and analyzing, as the two papers showing no difference identified Tregs as FoxP3+ cells and the paper showing a difference identified these cells as being CD25+FoxP3+[94-96]. This emphasizes the need for a robust gating strategy for the analysis of Tregs[97].

Additionally, the role of macrophages has been investigated. There are two major subtypes of macrophages, being the macrophages that support an effective antitumor response (M1) and the macrophages that promote tumor growth (M2). In the early development of CM, the M1-recruited macrophages shift to the M2 phenotype, thus favoring tumor proliferation and dissemination[98].

In contrast to CM, the pronounced infiltration of UM by immune cells is associated with a poor prognosis[99]. Primary UM with monosomy 3 is associated with infiltration with a variety of immune cells, including CD8+, CD4+, and CD3+CD8-FoxP3+ T cells as well as CD68+CD163+ M2 macrophages. The Class 2B tumors that display a gain in the copy number of chromosome 8q are associated with the increased expression of macrophage-attracting chemokines and a stronger influx of myeloid cells, whereas additional aberrations in BAP1 expression seem to drive T cell infiltration, irrespective of the chromosome 3 status[100]. The presence of a CD3+ immune infiltrate in Class 2 tumors, while nearly absent in Class 1 tumors, coincides with the increased gene expression of human leukocyte antigen (HLA), suggesting the local production of type II interferon[101]. Notably, the infiltration with all these immune cells is collectively increased, the balance of the different cells was of no clinical relevance[102,103], although one study suggested that the presence of the immunosuppressive Tregs within a subgroup of COX2+ primary UM forms an independent prognostic factor for worse overall survival[104].

1.2 Metastatic Melanoma

In many metastasized tumors, including CM, the presence of effector T lymphocytes is beneficial, including CD8+ T cells and CD4+ helper T cells. The presence of CD4+CD25+ Tregs may be detrimental[105]. Our group recently identified four intratumoral parameter profile that was associated with a better survival in metastatic CM patients. Namely, the presence of tumor infiltrating CD3+CD8+FoxP3− T cells, galectin-9+ dendritic cells (DC)/DC-like macrophages, a high CD14+CD163− (M1)/CD14+CD163+ (M2) macrophage ratio, and the expression of galectin-3 by tumor cells. Patients with three or four of the described parameters present displayed the longest overall survival[106].

Currently, one of the most established treatments for metastatic CM is via immune stimulation with checkpoint blockers. This type of treatment relies on antigen-specific T cell responses by alleviating tumor-induced immunoregulatory mechanisms[107]. Immune checkpoint blockade can achieve durable responses in many CM patients and has shown to improve overall survival in this patient group. The first blocking antibody that was tested and approved for the treatment of cancer patients was against cytotoxic T-lymphocyte antigen-4 (CTLA-4). CTLA-4 increases the activation threshold of T cells, reducing immune responses to weak antigens such as self- and tumor antigens. The second blocking antibody introduced into the clinic was targeting programmed death 1 (PD-1). While CTLA-4 mainly plays a role in the activation phase in the draining lymph node, PD-1 predominantly regulates the effector phase of T cell responses within peripheral tissues. PD-1 binding with its ligands decreases the magnitude of the immune response in T cells that are already engaged in an effector T cell response. This

results in a more restricted T cell activation compared to CTLA-4 blockade, which can lead to an unspecific activation of T cells in the lymphoid organs. This could explain why PD-1 inhibition shows fever side effects and greater antitumor activity than CTLA-4 inhibition[108-111]. The updated survival data from the CheckMate 067 study showed a 3-year overall survival of 58% in the patients treated with anti-PD-1 and anti-CTLA-4, 52% in patients with anti-PD-1 monotherapy and 34% in patients treated with anti-CTLA-4 monotherapy[108].

Treatment with these checkpoint blockers has been investigated in UM. Unfortunately, the clinical response rates reported for anti-PD-1 or anti-CTLA-4 are unimpressive, with no significant OS benefit in UM patients[112-120]. A trial investigating the combination of these checkpoint inhibitors is still ongoing (NCT01585194).

Little is known about the immune microenvironment of metastatic UM (mUM). Therefore, reasons underlying the poor response to immunotherapy are unclear and have led to speculation that UM may represent an immunotherapy resistant form of melanoma. Several recent findings might help to shed some light on why UM does not respond to immunotherapy like CM.

High mutational burden is predictive of the response to immune checkpoint inhibitors across multiple cancer types[121]. The neoantigens that derive from these tumor-specific mutations are potential targets for anti-tumor immune responses, as they are foreign to the immune system. Cutaneous melanoma is one of the tumors with the highest somatic mutation prevalence[122]. In contrast, UM lacks the UV-radiation mutation signature and has a low mean somatic mutation rate[123]. The lack of these targets could be a possible explanation as to why immune stimulation with checkpoint inhibitors alone is not sufficient in UM, while it can be sufficient in CM. However, low-mutational burden may also lead to the spontaneous activation of neoantigen-specific T cells[124,125].

In a recent pilot study, the immune profile of both CM and UM metastases was characterized. Overall, it seemed that the CD8 infiltration in both tumors was similar. Interestingly, the PD-1 expression levels were lower in mUM patients than those observed in metastatic CM (mCM). Furthermore, it also seemed that the expression of PD-L1 (one of the ligands of PD-1) was lower in the mUM group[126]. As activated tumor-reactive CD8+ T cells express PD-1, this may suggest that there either is a lack of tumor-antigen specific tumor infiltrating lymphocytes (TIL) in mUM or that they are locally suppressed by other means[127]. In the absence of a type 1 immune response, there is less interferon-gamma driven PD-L1 expression[128]. As the target for anti-PD-1 treatment is not expressed in most mUM patients, this provides another rationale for the lack of efficacy of anti-PD-1 treatment.

Preliminary data from an ongoing trial comparing the immune infiltrate of mUM and mCM show that in accordance with the previously mentioned trial, the density of CD3+CD8+, as well as the distance from CD8+ lymphocyte to tumor cell, was similar in both tumor types. However, macrophages were less numerous in mUM compared to mCM at baseline; further classification of these macrophages is still ongoing. Interestingly, the preliminary data also showed that enrichment for T cell and inflammatory gene expression was observed in a mUM patient with exceptional overall survival in contrast to an overall low CD8 and the absence of an immune gene expression profile in a patient with the shortest overall survival[129]. This suggests that some mUM are immunogenic, despite earlier reports on the immune infiltrate in primary UM. This notion is also supported by a recently published phase II clinical trial applying adoptive cell therapy to treat mUM patients. Twenty-one mUM patients were treated with autologous TIL. Of the 20 evaluable patients, seven (35%) achieved objective tumor regression (six partial response, one complete response), including mUM patients who had previously failed on anti-CTLA-4 and anti-PD-1 treatment. There was a strong correlation between clinical response, the autologous tumor reactivity of the infused TIL, and the number of reactive TIL infused. This clearly shows that despite the lack of an ultraviolet radiation signature, mUM do express antigens that are recognized by the adaptive immune system, suggesting that a lack of T cell activation in mUM is related to local immune suppression. Both biopsies prior and after TIL treatment were obtained from these patients, genomic and proteomic profiling is ongoing and whole exomic sequencing is being performed[130]. Despite the impressive overall response rate for patients with mUM, the durability was relatively short when compared to what has been observed in mCM. Moreover, a second phase II study is necessary, where patients with mUM are recruited with adoptive transfer of TIL to confirm the results in a larger cohort (NCT03467516).

Another potentially interesting cell-based therapy is treatment with chimeric antigen receptor (CAR) T cells. In hematological malignancies two CAR-T cell constructs targeting CD19 have been approved, both in the United States and in the European Union. One of the pilot trials currently recruiting melanoma patients uses c-Met as a target antigen (NCT03060356). As c-Met plays an important role in both CM and UM, this might be a promising treatment strategy for both melanoma subtypes.

Conclusions

Cutaneous and uveal melanoma both arise from melanocytes. However, they are biologically distinct tumor types. In recent years, many new treatment options have become available for patients with advanced cutaneous melanoma, improving the disease free and overall survival. Unfortunately, most of these new treatment options do not show the same responses in patients with metastatic uveal melanoma.

Chemokine receptors, which play a role in both tumor growth and the formation of metastases, have shown to be promising new targets. Based on the pre-clinical work with anti-CXCR4 and anti-IGF-1R, as well as the first clinical results with a MET/VEGF receptor inhibitor, several treatment options are now (further) investigated in the clinic. Multiple trials with both UM and CM patients that are treated with HDAC-inhibitors are also ongoing.

Recent studies indicate that the role of the adaptive immune system in primary versus metastatic UM might be very different. Where immune infiltrate in primary uveal melanoma is correlated with a worse overall survival, this difference was so far not seen in metastatic lesions. However, even when immune cells succeed in infiltrating metastatic UM lesions, these cells do not seem to be activated. Adoptive cell therapy trials in mUM indicate that metastatic UM are immunogenic and able to trigger tumor-reactive T cells; however, potentially, they are locally suppressed, similar to what is seen in primary UM.

As there is not yet a gold standard in the systemic treatment of metastatic UM, early detection and enrolment in clinical trials seems crucial.

Author Contributions

Conceptualization: M.K.v.d.K., S.H.v.d.B., and E.K.; writing—original draft preparation: M.K.v.d.K.; writing—review and editing: M.K.v.d.K., F.M.S., S.H.v.d.B., and E.K.; visualization: M.K.v.d.K.; supervision: E.K., and S.H.v.d.B.

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Conflicts of Interest

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CHAPTER
3

Immune checkpoint inhibitors in metastatic uveal melanoma

CHAPTER
3.1

Ipilimumab in Pretreated Metastatic Uveal Melanoma Patients. Results of the Dutch Working Group on Immunotherapy of Oncology (WIN-O)

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Sander Kelderman*, Monique K. van der Kooij*, Alfons J.M. van den Eertwegh, Patricia M.M.B. Soetekouw, Rob L.H. Jansen, Rob R.H. van den Brom, Geke A.P. Hospers, John B.A.G. Haanen, Ellen Kapiteijn, Christian U. Blank

*Joint first authors

To the Editor,

Uveal melanoma arises from melanocytes that reside in the iris, ciliary body or choroid of the eye. Local treatment can be divided into 'radical' enucleation and 'conservative' treatment. About 50% of patients develop metastasized disease and in up to 95% of these cases the liver is affected, due to the absence of lymphoid structures in the uvea. Once metastasized to the liver, surgical resection may be beneficial for small lesions, but less than 9% of patients fall into this category[1].

The blockade of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) by ipilimumab has become standard for pretreated patients with cutaneous melanoma based on a randomized phase III study[2]. This drug significantly improved the overall survival resulting in 20-25% of the patients still being alive after more than two years.

Due to its distinct biological and clinical nature (fast progression) uveal melanoma patients are often excluded from melanoma studies. Uveal melanoma patients have been allowed to be included in ipilimumab expanded access programs, in which some clinical activity has been described[3-6].

In our study, 22 pretreated metastatic uveal melanoma patients were treated homogenously with 3 mg/kg ipilimumab in the named patient program (NPP) by the Dutch immunotherapy working group (WIN-O) in the Netherlands. We describe here the toxicity and efficacy of ipilimumab at 3 mg/kg in a real world patient cohort of uveal melanoma patients.

Methods

Patients

Patients were treated by the Dutch immunotherapy working group (WIN-O) in an NPP of ipilimumab (NCT00495066) in which uveal melanoma patients were allowed to be included. Patients had to have unresectable, metastatic uveal melanoma (with or without brain metastases) and were required to have received at least one prior treatment regimen for metastatic disease. They had to be at least 16 years of age with a WHO performance status of 0, 1, or 2. A 28-day interval since the last treatment was required before inclusion. Evaluable patients that had given their written informed consent underwent radiologic evaluation of their tumor burden at baseline and at 12 weeks after their first ipilimumab course. The treatment protocol was approved by the local medical ethical committees.

Treatment

Ipilimumab was administered at 3 mg/kg in week 1, 4, 7 and 10. Prior to every infusion, hemoglobin, leucocytes and differentiation, platelets, liver function, renal function, thyroid and adrenal function were assessed for safety reasons and monitoring of toxicity. Immune-related adverse events (IrAEs) were scored using Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

Response and survival evaluation

At baseline and after four courses of ipilimumab at week 12, a computed tomography (CT) scan was made to evaluate the tumor response. We used the following radiological scoring systems; immune-related response criteria (irRC) and RECIST version 1.1. The response rates were termed as partial remission (PR) and complete remission (CR). BOR was also assessed using irRC to capture delayed anti-tumor responses often observed with immunotherapy. Clinical benefit was defined as the response proportion of patients plus SD lasting longer than 24 weeks. Estimates of OS and PFS were obtained using the Kaplan-Meier method.

Data-analysis

Data were retrospectively collected from all Dutch centers organized in the Dutch immunotherapy working group (WIN-O) participating in the Dutch expanded access program and having treated uveal melanoma patients (see also coauthors affiliations). Patients' data were retrospectively collected into a predefined SPSS database by each center individually. Descriptive statistics were performed using SPSS statistical software (version 17.0 for Windows, SPSS, Chicago, USA). The final data were graphed and analyzed using GraphPad Prism Version 5.0.

Results

Twenty-two metastatic uveal melanoma patients were treated in an NPP, which was open in the Netherlands from May 2010 until August 2011. The patient characteristics of this cohort are described in (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/0284186X.2013.786839). Median follow-up was 177 days (6.3 months). Twelve patients (55%) completed the four infusions of ipilimumab. Of the remaining 10 patients, nine had to discontinue treatment because of clinical deterioration due to disease progression (two of them died) and one because of severe adverse events (Figure 1).

In Table 1 the response to treatment is described. Of the 22 patients who received at least one ipilimumab infusion, 13 patients showed progressive disease (PD) and one patient had a PR. There was no SD or CR achieved according to RECIST 1.1. Eight patients were not evaluable (NE). Following irRC there were 12 patients with PD, one with SD, one with PR and no CRs.

TABLE 1 Response to treatment.

RECIST after 12 weeks	
Progressive disease	13 (59.1%)
Stable disease	0 (0%)
Partial response	1 (4.5%)
Complete response	0 (0%)
Not evaluable	8 (36.4%)
IRRC after 12 weeks	
Progressive disease	12 (54.5%)
Stable disease	1 (4.5%)
Partial response	1 (4.5%)
Complete response	0 (0%)
Not evaluable	8* (36.4%)
Best overall response	
Progressive disease	12 (54.5%)
Stable disease	1 (4.5%)
Partial response	1 (4.5%)
Complete response	0 (0%)
Not evaluable	8* (36.4%)
Clinical benefit (based on BOR)	
PD/NE	20 (90.9%)
SD > 24w/PR/CR	2 (9.1%)
Response rate (based on BOR)	
SD/PD/NE	21 (95.5%)
CR/PR	1 (4.5%)

*Not evaluable due to fast disease progression and death within 65 days after start of treatment.

At the time of manuscript preparation one patient (4.5%) was still alive with ongoing SD (+ 16 months). The patient observing a PR was eligible for ipilimumab reinduction

due to disease progression seven months after ipilimumab initiation. Unfortunately, the reinduction did not result in a renewed response.

The OS and PFS curves of our 22 patients are depicted in Figure 1. The Kaplan-Meier analyses show a median PFS of 2.9 months. The median OS was 5.2 months with a one-year survival of 27%.

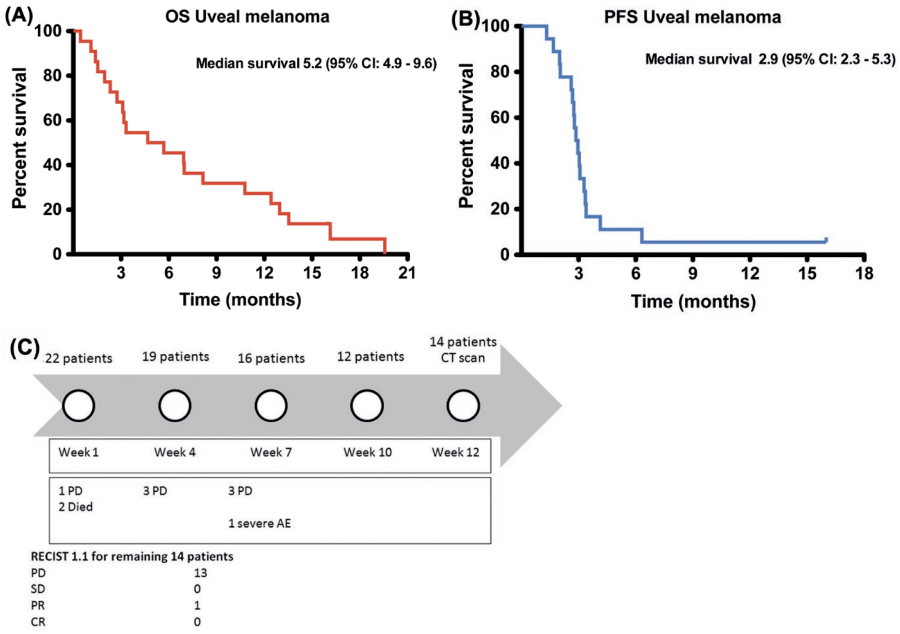

FIGURE 1 Overall survival (OS) and progression free survival (PFS) of uveal melanoma patients treated with ipilimumab 3 mg/kg. All uveal melanoma patients treated in the Dutch expanded access program were evaluated retrospectively for OS (**A, red**) and PFS (**B, blue**) All 22 patients were included for PFS analysis, and the patients not evaluable at week 12 were defined to be progressive at the date of clinical deterioration. The detailed follow-up of the patients during treatment is shown in **C**.

As shown in Supplementary Table II (online at http://informahealthcare.com/doi/abs/10.3109/0284186X.2013.786839) most adverse events were immune-related. Here, we only describe the grade 3 irAEs, as grade 1 or 2 was not considered clinically relevant. Grade 3 colitis was seen in two patients. One patient developed grade 3 hepatitis. All patients received corticosteroid treatment (1 mg/kg prednisolon) after which irAEs quickly resolved.

Discussion

In our study, 22 M1c uveal melanoma patients were treated by the Dutch immunotherapy working group (WIN-O) in an ipilimumab NPP in the Netherlands.

Only 12 patients (55%) completed the treatment course consisting of four infusions of ipilimumab at the dose of 3 mg/kg. Within the cohort of the 22 patients, only one patient had a PR according to RECIST and another patient had SD according to irRC.

In another recently published study performed by Danielli et al., nine of 13 patients (69%) completed the course of four infusions and two patients showed SD that remained until week 36[4]. Median OS was 36 weeks (9 months), in contrast to 21 weeks (5.2 months) in our cohort.

Three other, so far unpublished, retrospective analyses have evaluated the efficacy of ipilimumab in uveal melanoma patients. A single center analysis of 20 uveal melanoma patients treated at the Memorial Sloan-Kettering Cancer Center observed within a group of 20 patients that received a median of four infusions of ipilimumab (20%) two PRs (one at week 12 and one at week 24) and seven SD. This resulted in a median survival of 8.6 months (95% CI 3.5-NR), with two ongoing PRs (3 + yrs and 24 + wks)[3]. The other expanded access programs, the Italian and the US, observed a one-year OS rate of 32% and 34%, respectively, which were comparable to the one-year OS rate observed in our study (27%)[5,6].

Furthermore, initial phase I studies indicated a correlation between the presence of grade 3-4 irAEs and response[7], that was not confirmed in the phase III studies[2,8]. Similarly, no such correlation was found in our analysis.

In conclusion, our retrospective analysis from the Dutch expanded access program indicates limited clinical activity of ipilimumab in pretreated patients with metastatic uveal melanoma at a dose of 3 mg/kg. Currently, two single-arm phase II clinical trials are testing ipilimumab in uveal melanoma patients (www.clinicaltrials.gov Identifier: NCT01355120 and NCT01034787). In addition, a phase Ib/II study exploring the combination of ipilimumab with radiofrequency ablation (RFA) in uveal melanoma patients has been started recently at the Netherlands Cancer Institute (NKI-AVL), Amsterdam (www.trialregister.nl Identifier: NTR3488). Intensive patient characterization and biomarker research in these studies will hopefully be able to identify predictive factors for response and survival to targeted therapy and immunotherapy in metastatic uveal melanoma.

Conflicts of interest

The authors alone are responsible for the content and writing of the paper.

A.J.M. Van Den Eertwegh, G.A.P. Hospers, E. Kapiteijn and C.U. Blank have received compensation for participation in advisory board meetings of BMS Netherlands. C.U. Blank receives funding for an investigator initiated study from BMS

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CHAPTER
3.2

Anti-PD-1 Treatment in Metastatic Uveal Melanoma in the Netherlands

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Monique K. van der Kooij, Arjen Joosse, Frank M. Speetjens, Geke A.P. Hospers, Kees Bisschop, Jan Willem B. de Groot, Ruther Koornstra, Christian U. Blank, Ellen Kapiteijn

To the Editor,

Uveal melanoma (UM) is a rare type of melanoma, with an incidence of 4.4 cases per million in Europe each year[1]. During recent years, different treatment approaches have been tested in patients with metastatic UM. Responses have been reported mainly with localized treatment in patients with a limited number of metastases in the liver[2-6]. When diffuse liver involvement and/or extrahepatic disease have developed, systemic therapies are warranted. So far, systemic therapies such as targeted therapy with selumetinib[7] or classic chemotherapy[8] have failed in metastasized UM.

During the past three years, the European Medicines Agency and the Food and Drug Administration (FDA) have approved three immune checkpoint inhibitors for the treatment of melanoma; ipilimumab (a monoclonal antibody targeting cytotoxic T-lymphocyte-associated protein 4, anti-CTLA-4), pembrolizumab and nivolumab (both programed cell death protein 1 antibodies, anti-PD-1). Previous retrospective studies in metastatic UM with ipilimumab did not yield the same positive results as in cutaneous melanoma[9-11].

Here, we present the clinical outcome of 17 metastasized UM patients treated with nivolumab or pembrolizumab in the Netherlands.

Methods

Patients

Some of the patients were treated in a named patient program (NPP) according to inclusion criteria. Other patients were treated outside this NPP, following clinical criteria of the treating physician. In all 17 patients this meant that they were ≥18 years of age, were diagnosed with unresectable metastatic UM, had a reasonable performance score (WHO performance status of 0-2) and adequate organ and bone marrow function. Patients did not require previous ipilimumab treatment. Patients with central nervous system metastases had to be clinically stable before enrollment.

Treatment

Patients were treated with respectively 2 mg/kg pembrolizumab intravenously every three weeks or 3 mg/kg nivolumab intravenously every two weeks. Treatment beyond disease progression was allowed, provided that the patient had clinical benefit and no severe adverse effects. Before every administration the patients' blood was tested, as completed per clinical practice for at least lactate dehydrogenase, liver, kidney, bone marrow and thyroid function.

Response and progression-free survival evaluation

Imaging was performed at baseline, and every 12 weeks and at the investigators' discretion. A computed tomography (CT) scan was made to evaluate the tumor response according to the radiological scoring system Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1[12]. Estimates of overall (OS) and progression-free survival (PFS) were obtained using the Kaplan-Meier method.

TABLE 1 Patient characteristics

Age	Sex	WHO	Infusions	Lesion sites at start	Therapies*
51	Female	2	1	Lu, Ma, Cor, LN, Mu, Sp, Bo	3
68	Female	1	3	Li (multiple)	2
68	Female	1	1	Lu, Li, LN, Th, Pt, SC	4
40	Male	0	7	LN, Ad, Pt, Sp, SC	2
60	Female	0	6**	Lu, Li, Bo, SC	1
69	Male	0	6**	Li	1
45	Female	0	4	Li (multiple)	1
44	Female	0	3	Lu, Li	1
49	Female	0	4	Lu, Li, SC, LN	0
28	Male	0	2	Li (multiple)	0
72	Female	0	8 ongoing	Lu, Li	0
54	Male	0	2	Li, LN, Ad, Bo, Pl	0
73	Female	0	5	Li (multiple)	2
67	Female	0	6 ongoing	Li	0
68	Female	0	4 ongoing	Lu, Li	0
49	Female	0	4 ongoing	Lu, Ad, LN, Sp, Bo	0
63	Male	-	3	Lu, Li, LN, Bo, SC	0

* Number of prior systemic therapies
** Patients received nivolumab
Ad: adrenal; Bo: bone; B: brain; Li: liver; LN: lymph node; Lu: lung; Ma: mammae; Mu: muscle; Pl: pleurae; Pt: peritoneal; SC: subcutaneous; Sp: spleen; Th: thyroid.

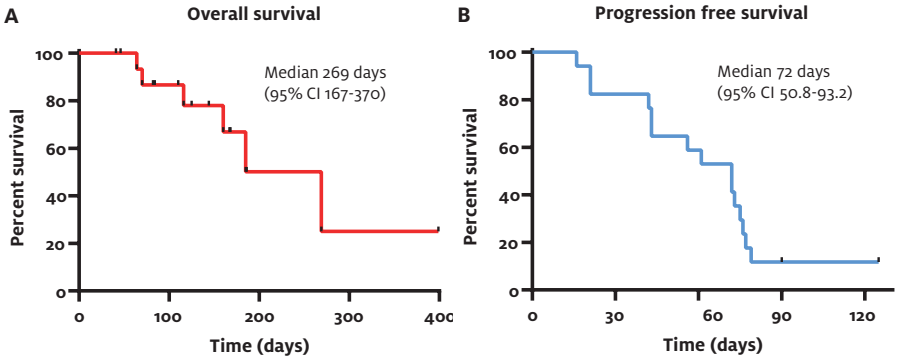

FIGURE 1 Progression-free (PFS) and overall survival (OS) of uveal melanoma patients treated with either pembrolizumab or nivolumab. OS is shown in (**A**), with 11 patients still alive at time of manuscript preparation. PFS is depicted in (**B**), with two patients having stable disease at time of manuscript preparation.

Data analysis

Data were retrospectively collected from Dutch centers organized by the Dutch Immunotherapy Working Group (WIN-O). These data were collected into a predefined database, which was closed on 4 August 2016. Descriptive statistics were performed using SPSS (Version 23 for Windows, SPSS, Chicago, IL, USA).

Results

Seventeen metastatic UM patients were treated with anti-PD-1 in five different medical centers in the Netherlands between June 2014 and July 2016. The characteristics of this cohort are described in Table 1. The median follow-up was four months. In 10 patients (58.8%) at least four infusions of anti-PD-1 were completed and a CT scan to evaluate tumor response was performed. In three patients a CT scan was performed after three courses of anti-PD-1 after which treatment was discontinued due to fast progressive disease. The remaining four patients (23.5%) deteriorated due to progressive disease too fast to be evaluated by CT scans (two patients after one course of anti-PD-1 and two patients after two courses).

Of the 17 patients who received at least one anti-PD-1 infusion, 15 had progressive disease either clinically or on CT scan (six of them died during or shortly after discontinuing treatment). No patient experienced grade 3 or 4 adverse events. One patient experienced grade 2 toxicodermia which was treated with topical steroids.

At the time of database closure, two of the 15 patients with progressive disease had clinical benefit in terms of symptom reduction and underwent further treatment

with anti-PD-1. Two patients (11.8%) were alive and on treatment with ongoing stable disease; with both patients having received four courses at the time of manuscript preparations. Figure 1(a) demonstrates OS (median 9.6 months) and PFS (median 2.3 months) of our 17 patients is shown in Figure 1(b).

Discussion

In our study, 17 metastasized UM patients were treated with anti-PD-1 (either nivolumab or pembrolizumab). Four patients were continuing anti-PD-1 treatment at the time of manuscript preparations; two patients because of clinical benefit in terms of symptom reduction and two patients due to ongoing stable disease.

In another recent study by Kottschade et al., a total of 10 UM patients were treated with pembrolizumab. The median PFS was 18 weeks with four patients still ongoing treatment, which is high compared to the 10.3 weeks (2.3 months) in our cohort, indicating strong patient selection[13]. Our study differed from the research by Kottschade et al. because we included patients with WHO performance score of 2. Furthermore, we also included treatment-naive patients, whereas Kottschade et al. only included patients who were progressive on treatment with ipilimumab. Moreover, the number and location of metastases was not described by Kottschade et al.

In another recent study from Algazi et al., a total of 56 patients were treated with a PD-1 or PD-L1 antibody. The median PFS was 2.6 months and the median OS was 7.6 months, which is comparable to the median PFS in our study of 2.3 months and the median OS of 9.6 months[14]. Algazi et al. concluded that PD-1 and PD-L1 antibodies rarely confer durable remissions in patients with metastatic UM.

Forthcoming are the results from a phase II trial with pembrolizumab in patients with metastasized UM (NCT02359851) and two phase II studies investigating the combination of ipilimumab with nivolumab in treatment-naive UM patients (NCT02626962) or patients with any number of prior treatments (NCT01585194).

The OS data of our study should be interpreted with caution. Limitations of these data include the small sample size of 17 patients, the short follow-up period, differences in prior treatment and subsequent treatments received.

In conclusion, our retrospective analysis of 17 metastatic UM patients treated with anti-PD-1 in the Netherlands indicates limited clinical activity. Overall, this is in agreement with the recently published study by Algazi et al. and is in contrast with the more favorable response reported by Kottschade et al. More studies are needed to explore combination therapies of checkpoint inhibitors, targeted and immunotherapy,

or local therapies and checkpoint inhibitors for metastasized UM patients to improve prognosis in this patient group.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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CHAPTER
4

Metastatic Uveal Melanoma: Treatment Strategies and Survival – Results From the Dutch Melanoma Treatment Registry

Cancers 2019; 11, 1007

Anouk Jochems, Monique K. van der Kooij, Marta Fiocco,
Maartje G. Schouwenburg, Maureen J. Aarts, Alexander C. van Akkooi,
Franchette W.P.J. van den Berkmortel, Christian U. Blank,
Alfonsus J.M. van den Eertwegh, Margreet G. Franken, Jan Willem B. de Groot,
John B.A.G. Haanen, Geke A.P. Hospers, Rutger H. Koornstra, Wim H.J. Kruit,
Marieke Louwman, Djura Piersma, Rozemarijn S. van Rijn,
Karijn P.M. Suijkerbuijk, Albert J. ten Tije, Gerard Vreugdenhil, Michel W.J.M. Wouters,
Michiel C.T. van Zeijl, Koos J.M. van der Hoeven, Ellen Kapiteijn

Abstract

Uveal melanoma (UM) is the most common primary intraocular tumor in adults. Up to 50% of UM patients will develop metastases. We present data of 175 metastatic UM patients diagnosed in the Netherlands between July 2012 and March 2018. In our cohort, elevated lactate dehydrogenase level (LDH) is an important factor associated with poorer survival (Hazard Ratio (HR) 9.0, 95% Confidence Interval (CI) 5.63-14.35), and the presence of liver metastases is negatively associated with survival (HR 2.09, 95%CI 1.07-4.08). We used data from the nation-wide Dutch Melanoma Treatment Registry (DMTR) providing a complete overview of the location of metastases at time of stage IV disease. In 154 (88%) patients, the liver was affected, and only 3 patients were reported to have brain metastases. In 63 (36%) patients, mutation analysis was performed, showing a GNA11 mutation in 28.6% and a GNAQ mutation in 49.2% of the analyzed patients. In the absence of standard care of treatment options, metastatic UM patients are often directed to clinical trials. Patients participating in clinical trials are often subject to selection and usually do not represent the entire metastatic UM population. By using our nation-wide cohort, we are able to describe real-life treatment choices made in metastatic UM patients and 1-year survival rates in selected groups of patients.

Introduction

Uveal melanoma (UM) is the most common primary intraocular tumor in adults and arises from the melanocytes residing in the stroma[1,2]. Between 2012 and 2018, the incidence of primary uveal melanoma was approximately 200 new cases per year in the Netherlands[3]. European data on the incidence of primary uveal melanoma report 4.4 cases per million in Europe[4]. Among all intraocular melanomas, choroidal melanomas occur most frequently (80-90% of cases), but tumors may also develop in the iris or ciliary body[2]. The diagnosis of uveal melanoma is based on non-invasive testing techniques, such as fundoscopy or ultrasound, performed by an experienced clinician. Ocular treatment of uveal melanoma consists of enucleation ("radical treatment") or radiotherapy, usually in the form of plaque brachytherapy or proton radiotherapy ("conservative treatment")[5]. Management of primary uveal melanoma is guided by the size and location of the tumor, presence of extraocular extension, visual potential and patient age and preference. In selected patients, both treatment modalities show similar survival and risk of metastases, with radiotherapy having the advantage of a better cosmetic result and the possibility of saving vision in the smaller tumors[6].

Unfortunately, up to 50% of patients with uveal melanoma will ultimately develop metastatic disease. The most frequently affected metastatic site is the liver[4,6,7]. The site of the metastases has an impact on survival; patients with liver metastasis have a poorer prognosis than patients with extrahepatic metastasis[8,9]. Previously, it was thought that there would be no survival advantage in early diagnosis of metastatic disease because of the lack of standard of care therapy for metastatic uveal melanoma. However, patients with early diagnosis of metastatic disease might benefit from liver-directed therapy, which is associated with clinical utility[10-15] or they might benefit from participation in a clinical trial. Under the Dutch and UK uveal melanoma guidelines[16,17], patients with primary uveal melanoma are therefore advised to have 6-monthly liver function tests in combination with liver-specific imaging by a non-ionizing modality to detect metastatic disease in an earlier phase.

On a molecular level, uveal melanomas differ significantly from cutaneous melanomas. Unlike cutaneous melanoma, uveal melanoma is not characterized by frequent BRAF or NRAS mutations, so that advances in targeted therapy for cutaneous melanoma are not applicable to metastatic uveal melanoma. Early activating mutations in GNAQ or GNA11 are present in about 80% of primary uveal melanomas. These lead to activation of downstream signaling pathways[18]. Inactivating somatic mutations are present in the gene encoding BRCA1-associated protein 1 (BAP1) in more than 80% of metastasizing tumors, implicating a role in the progression of uveal melanoma.[19] Mutations in SF3B1 and EIF1AX in primary uveal melanoma are associated with a

relatively good prognosis[20,21]. Greater understanding of the molecular pathogenesis may provide opportunities for patients who benefit from surveillance and may eventually provide specific targeted therapy for metastatic uveal melanoma patients.

Over the past few years, different treatment strategies have been evaluated in patients with metastatic uveal melanoma. The best responses have been reported with local treatment strategies in patients with exclusive and limited hepatic metastasis in whom surgical resection, isolated hepatic perfusion with melphalan, radiotherapy, radiofrequency ablation or radio-embolization was performed[10-15]. In patients with diffuse liver metastases or extensive extrahepatic metastases, systemic therapy is the only treatment strategy available. Several combinations of drugs have been investigated in phase Ib/II/III trials in patients with metastatic uveal melanoma. Until now, none of the systemic treatments with chemotherapy[22-24], immune checkpoint inhibitors[25-32] or targeted therapy[33,34], have shown substantial efficacy in metastatic uveal melanoma.

In this article, we present data from our Dutch cohort of metastatic uveal melanoma patients describing affected metastatic sites, mutation analysis, clinical characteristics associated with survival and treatment choices made and the corresponding one-year survival. By describing these groups of patients, we show the impact of clinical characteristics and selecting metastatic UM patients for treatment in our real-life population.

Patients and Methods

Data source

Since 2013, all Dutch metastatic melanoma patients have been referred to one of the 14 melanoma expert centers in the Netherlands. This centralization of metastatic melanoma patients and the registration in the Dutch Melanoma Treatment Registry (DMTR), providing nation-wide coverage retrospectively starting from July 2012, was initiated to assure safety and quality of melanoma care in the Netherlands[36]. Since the DMTR was set up, all patients with metastatic melanoma have been included in the registry, irrespective of the type of primary melanoma (i.e., cutaneous, uveal, or mucosal melanoma). The DMTR provides aggregated data information on basic patient and tumor characteristics, treatment regimens, grade 3 and 4 treatment related adverse events (according to the Common Terminology Criteria for Adverse Events, version 4.0) and clinical outcomes.

In compliance with Dutch regulations, the DMTR was approved by a medical ethical committee (METC Leiden University Medical Center, 3 September 2013) and is not

considered subject to the Medical Research Involving Human Subjects Act. All data are collected anonymously and only aggregated data are available for research and quality improvements. Data extraction from medical files is performed by data-employees. No informed consent will be signed, but patients are offered an opt-out possibility if they do not want their data registered in the DMTR. For this study, the data cut-off date was 25th March 2018.

Patients

Between July 2012 and March 2018, 227 patients with metastatic uveal melanoma were registered in the DMTR. Patients who received treatment before the DMTR was set up were excluded from analysis (Figure 1). We analyzed 175 treatment-naive patients according to the type of treatment initiated at first presentation with metastatic disease: i.e., patients could be receiving: (i) systemic therapy, (ii) local treatment, or (iii) no tumor-directed therapy, but best supportive care (BSC). For this manuscript, we analyzed only patients who had their first treatment post July 2012.

Systemic therapy included a variety of regimens with chemotherapy, immune checkpoint inhibitors and targeted drugs. Local treatment strategies included surgical resection, isolated hepatic perfusion with melphalan, radiotherapy, radiofrequency ablation or immune-embolization. Treatment strategies were performed either as standard care or in the context of participation in a clinical trial.

Statistical Analysis

Descriptive statistics were employed to summarize patient baseline characteristics on registration in the DMTR. To test the difference between categorical variables for different treatment strategy groups, a chi-square test was applied (Table 1). A rank-sum test has been used to test the difference between the median time from diagnosis to stage IV disease between groups of patients. Survival from the diagnosis of metastatic disease, was estimated according to Kaplan-Meier's method. Median follow up was computed with reverse Kaplan-Meier method[39].

A univariable Cox analysis using variables "age" (age as a continuous variable), "gender" (male versus female), "WHO performance score" (WHO 0-1 vs. WHO $\geq$ 2), "LDH level" (elevated vs. non-elevated LDH) and the "presence of liver metastases" was performed. Subsequently, a multivariable Cox regression model was estimated, including the variables known to influence survival in metastatic cutaneous melanoma patients. All statistical analyses were conducted using SPSS (SPSS, version 23, IBM Corp. released 2015, Armonk, NY, USA).

Results

Patient Characteristics

Of the 3959 registered patients in the DMTR, a total of 175 metastatic uveal melanoma patients were identified for analysis (Figure 1). Baseline characteristics are presented in Table 1.

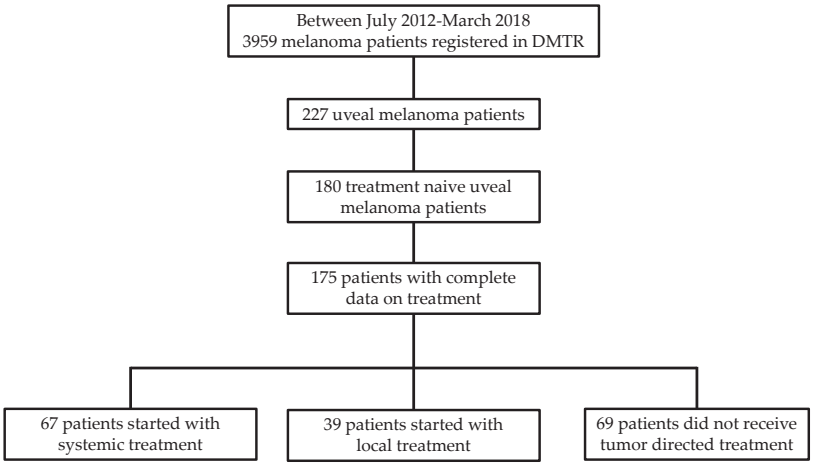

FIGURE 1 Nation-wide cohort of metastatic uveal melanoma patients registered in the Dutch Melanoma Treatment Registry (DMTR): All patients with complete data on treatment were analyzed and subdivided based on the first treatment option when diagnosed with metastatic disease.

The median age of metastatic UM patients in this cohort was 65 years. The majority of patients (74.9%) scored well on the World Health Organization (WHO) performance scale (0-1). Lactate dehydrogenase level (LDH) was elevated in 85 (48.6%) patients (Table 1). The liver was the most affected site: 88% of patients having liver metastases. Other affected sites were the lungs (25.1%), lymph nodes (16%) and bones (15.4%) (Figure 2). Differences in clinical characteristics between the treatment groups are presented in Table 1.

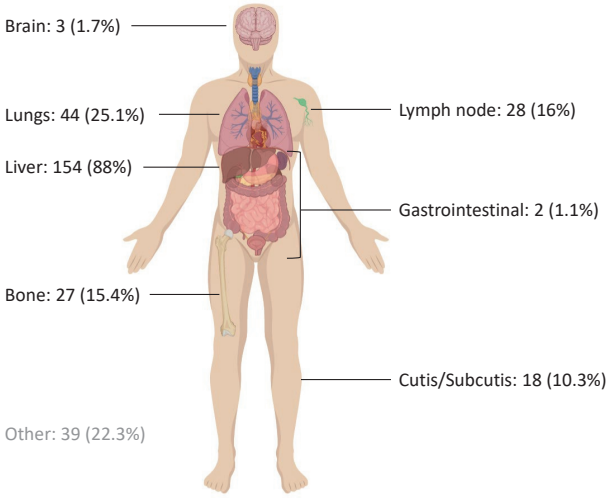

FIGURE 2 Frequency of affected organ in our cohort of patients with metastatic uveal melanoma (more than one organ can be affected).

Mutation Analysis

Molecular analysis of the activating mutation in the GNAQ or GNA11 genes was performed in 63 patients (36%) (Figure 3). The fact that detection of these mutation was of no therapeutic consequence might explain why these genes were not included in a standard NGS panel. In 31 of these 63 (49.2%) patients a mutation in the GNAQ was discovered and in 18 patients (28.6%) a GNA11 mutation was confirmed. These results are consistent with the known literature describing most primary uveal melanoma having a GNAQ or GNA11 mutation[18,35].

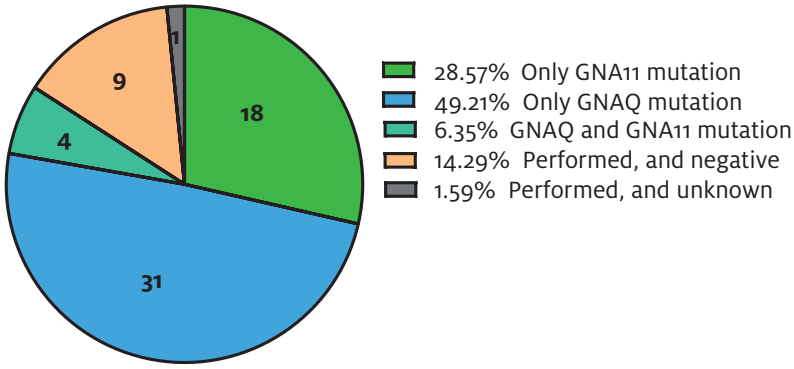

FIGURE 3 Results of molecular analysis of GNAQ/GNA11 mutation. Analysis was performed in 63 of 175 patients (36%).

TABLE 1 Patient characteristics at the moment of diagnosis with metastatic disease.

Patient Characteristics	All patients (n = 175)	Systemic Therapy (n = 67)	Local Therapy (n = 39)	Best Supportive Care (n = 69)	p-value
Age					0.001
Median, years (range)	65 (29-89)	61 (29-80)	61 (41-80)	69 (45-89)	
<65 years (%)	86 (49.1)	39 (58.2)	25 (64.1)	22 (31.9)	
≥65 years (%)	89 (50.9)	28 (41.8)	14 (35.9)	47 (68.1)	
Gender (%)					0.98
Male	88 (50.3)	34 (50.7)	20 (51.3)	34 (49.3)	
Female	87 (49.7)	33 (49.3)	19 (48.7)	35 (50.7)	
WHO performance score (%)					0.000
0	106 (60.6)	55 (82.1)	21 (53.8)	30 (60.6)	
1	25 (14.3)	7 (10.4)	3 (7.7)	15 (21.7)	
2	11 (6.3)	2 (3)	1 (2.6)	8 (11.6)	
3	3 (1.7)	0	0	3 (4.3)	
4	1 (0.6)	0	0	1 (1.4)	
Unknown	29 (16.6)	3 (4,5)	14 (35,9)	12 (17.4)	
Median time from diagnosis primary tumor to stage IV months (range)	38 (0-477)	43 (0-296)	29 (0-477)	42 (0-361)	0.02 *
Brain metastases (%)					
No	169 (96.6)	64 (95.5)	39 (100)	66 (95.7)	
Yes	3 (1.7)	2 (3)	0	1 (1.4)	

Unknown	3 (1.7)	1 (1.5)	0	2 (2.9)	
Liver metastases (%)					0.10
No	20 (11.4)	11 (16.4)	1 (2.6)	8 (11.6)	
Yes	154 (88)	56 (83.6)	38 (97.4)	60 (87)	
Unknown	1 (0.6)	0	0	1 (1.4)	
Metastatic sites (%)					0.002
<3 metastatic sites	134 (76.6)	44 (65.7)	39 (100)	52 (75.4)	
≥3 metastatic sites	31 (17.7)	18 (26.9)	0	13 (18.8)	
Unknown	10 (5.7)	5 (7.5)	0	4 (5.8)	
LDH (%)					0.000
Not elevated	81 (46.3)	37 (55.2)	26 (66.7)	18 (26.1)	
Elevated (250-500)	34 (19.4)	12 (17.9)	9 (23.1)	13 (18.8)	
Elevated (>500)	51 (29.1)	17 (25.4)	1 (2.6)	33 (47.8)	
Unknown	9 (5.1)	1 (1.5)	3 (7.7)	5 (7.2)	

* A rank-sum test for the median time from diagnosis to stage IV disease was used to test the difference between groups of patients. WHO performance score: World-Health Organization performance score. LDH: lactate dehydrogenase.

Treatment of Metastatic UM Patients

In our study, 67 patients (38.3%) received systemic therapy when diagnosed with metastatic disease. Several systemic drug regimens were applied, both in- and outside a clinical trial setting as there is no standard of care for patients with metastatic uveal melanoma. These regimens consisted of chemotherapy with dacarbazine, immune checkpoint inhibitors or targeted drugs. Several different clinical trials, varying from phase I to phase III trials, were open for patient enrollment at different time windows in the investigated period. All patients receiving a targeted drug participated in a clinical trial; for example, in the NCT01430416 trial (phase 1 trial with AEB071), NCT01801358 trial (phase 1b/II study with AEB071 + MEK162), NCT01974752 trial (phase 3 trial with selumetinib), or NCT02601378 (phase 1 trial with LXS196). In addition, patients could be included into the N11RFA trial, a phase II study exploring the combination of ipilimumab with RFA. Fifty-three (79.1%) of 67 patients were treated in a clinical trial as a part of first-line systemic therapy. Some patients received more than one treatment after the failure of first-line therapy. During registration, a total of 108 systemic therapies were given, in total 85 (78.7%) of these treatments were part of participation in a clinical trial (Figure 4).

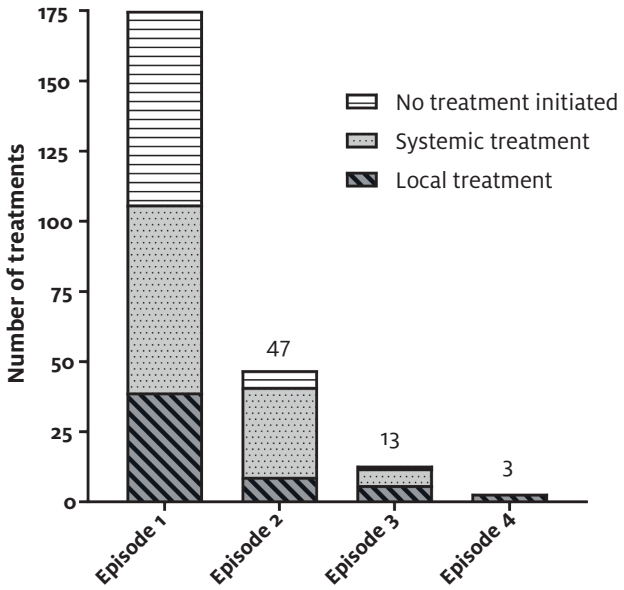

FIGURE 4 Treatment strategies per treatment episode. Some patients received more than one line of treatment after failure of first-line treatment. (treatment episode 1: treatment strategy performed when diagnosed with metastatic uveal melanoma, treatment episode 2: second treatment strategy after failure of first-line treatment etc.).

Sixteen patients received systemic treatment with a checkpoint inhibitor outside a clinical trial setting. Four patients received the anti-CTLA-4 antibody ipilimumab and 12 patients received an anti-PD-1 antibody. One patient was treated with the combination of anti-CTLA-4 and anti-PD-1 therapy. As most patients treated with anti-CTLA-4 antibody were included in a clinical trial (as part of a phase II study exploring the combination of ipilimumab with RFA, EudraCT Number: 2011-004200-38), overall survival data for this group are not yet available. The median OS of these 12 patients treated with an anti-PD-1 antibody was 54.3 weeks, ranging between 6 and 104 weeks. Data on duration of treatment, best overall response and overall survival are shown in Figure 5. Median follow-up computed with reverse Kaplan-Meier was equal to 89 weeks (95% CI 70.76-107.24).

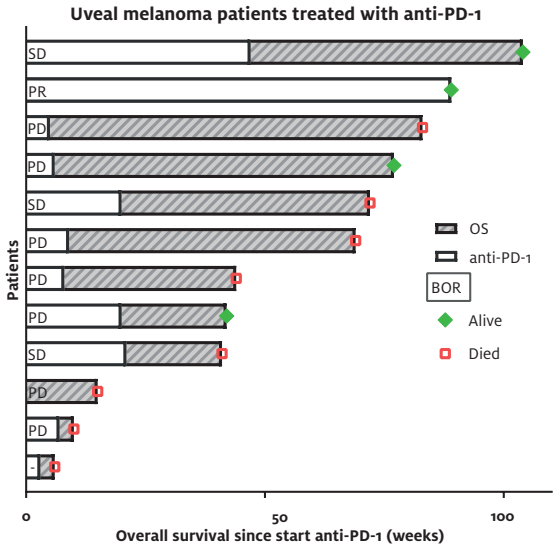

FIGURE 5 Best response and survival of 12 metastatic UM patients treated with an anti-PD-1 antibody (no clinical trial participation).

Thirty-nine patients (22.3%) received local treatment when first diagnosed with metastatic uveal melanoma. These local treatment regimens included surgical resection of metastases, isolated hepatic perfusion with melphalan, radiotherapy, radiofrequency ablation or radio-embolization. Sixty-nine patients (39.4%) did not receive anti-tumor directed therapy but received best supportive care (Figure 1).

Survival

The median follow-up was computed with reverse Kaplan-Meier (where the event indicator is reversed so that the outcome of interest is censored[36]) and was equal to

120 weeks (95% CI 96.3-143.7). One year after the diagnosis of metastatic uveal melanoma, 47.8% of all patients were alive (95% CI 40.4-55.2). There is a considerable difference in survival at one year among patients belonging to different treatment groups and patients included in the BSC-group. The prognosis at one-year observed in patients receiving systemic therapy or local therapy was 49% (95% CI 37-61) and 82.1% (95% CI 70.1-94.1), respectively. One-year survival for patients receiving best supportive care was equal to 27.5% (95% CI 16.9-38.1) (Figure 6).

The multivariable Cox analysis showed that slight to moderately elevated LDH (250-500 U/L) and high LDH level (>500 U/L) were a statistically significant factor associated with poor survival (p <0.001), HR of 1.8 (95% CI 1.07-3.01) and 9.0 (95% CI 5.63-14.35) respectively. Also, the presence of liver metastases was negatively associated with survival, HR 2.09 (95% CI 1.07-4.08, p = 0.03). A WHO performance score >1 on its own seemed to be associated with poorer survival in a univariable Cox analysis. However, when included in the multivariable analysis this association was no longer statistically significant. "Age" as a continuous variable was included in the model, but was not statistically significant (HR 1.0 (95% CI 0.99-1.02), p = 0.69) (Figure 7).

Figure S1 shows Kaplan-Meier estimates for survival when patients are categorized according to non-elevated versus elevated serum LDH for all three treatment groups at baseline. Both in the group of patients not receiving tumor-directed treatment (BSC) and the systemically treated group, an LDH above 250 U/l was clearly associated with poorer survival (p <0.001). However, in the local treatment group, this difference was not statistically significant (p = 0.15).

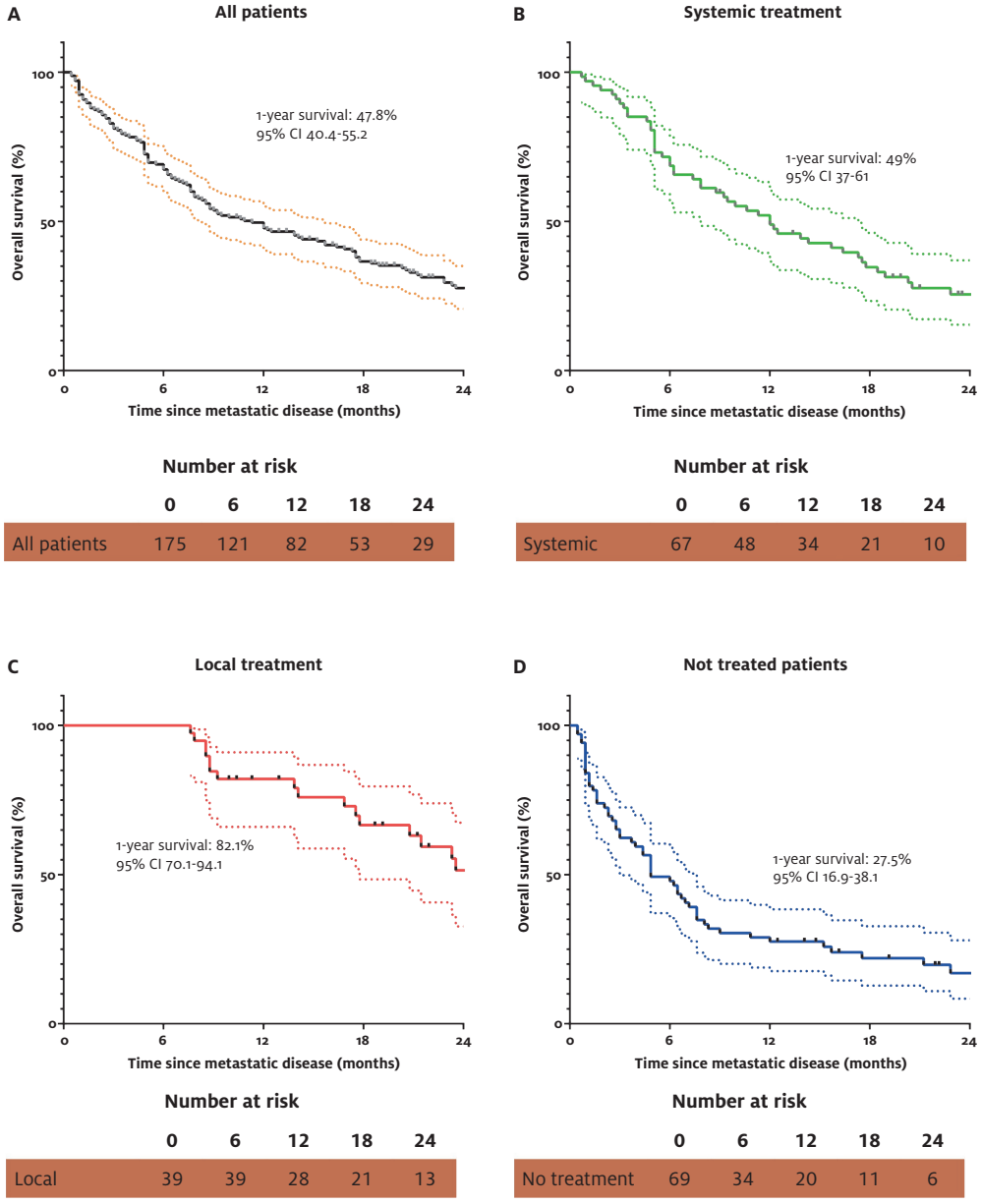

FIGURE 6 Kaplan-Meier Estimates for all 175 metastatic UM patients and per treatment strategy administered when diagnosed with metastatic disease. (**A**) Kaplan-Meier (KM) estimate for all metastatic UM patients, (**B**) KM estimate for patients treated with systemic therapy, (**C**) KM estimate for patients with local treatment, (**D**) KM estimate for patients receiving no tumor directed treatment (best supportive care).

Variable	Reference group	HR	95% CI
Age ≥65 years	Age <65 years	1.0	0.99-1.02
WHO 2, 3, 4	WHO 0 or 1	1.6	0.83-3.13
WHO unknown	WHO 0 or 1	1.2	0.74-1.92
LDH 250-500 U/l	Normal LDH	1.8	1.07-3.01
LDH >500 U/l	Normal LDH	9.0	5.63-14.35
LDH not determined	Normal LDH	1.65	0.69-3.90
Liver metastases	No liver metastases	2.09	1.07-4.08

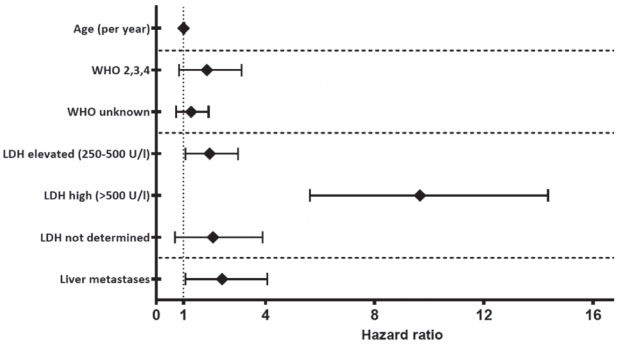

FIGURE 7 Multivariable hazard ratios (HR) associated with poorer survival in the full cohort along with the 95% confidence interval (CI).

Discussion

Metastatic uveal melanoma has a poor prognosis, usually leading to rapid clinical decline and early death. According to the literature, the majority of patients survive for less than 12 months[7,8]. In our cohort, we analyzed 175 patients with metastatic uveal melanoma according to first-line treatment strategies administered when they were diagnosed with stage IV disease between July 2012 and March 2018. The real-world results of this observational cohort are a reflection of uveal melanoma care available in the Netherlands and this article does not compare different treatment strategies and/or the impact on patient outcome. In our cohort, one-year survival for all patients with metastatic uveal melanoma is equal to 47.8% (95% CI 40.4-55.2), similar to that reported in known publications[7,8]. Studies reporting on survival in metastatic uveal melanoma have found the best results in terms of survival among patients in whom surgery or ablative procedures can be performed and among patients with solitary hepatic metastases[10-15]. Overall, these findings are suggestive of survival benefit, although it is likely that there is a selection bias towards the most clinically fit patients[9]. Based on the results in literature, the first choice of treatment in the Netherlands is, whenever possible, surgery, ablative procedures or isolated hepatic perfusion with melphalan (in a clinical trial setting). In line with the literature, our cohort shows a selection of relatively younger patients, with good WHO performance score, fewer metastatic sites and less elevated LDH who were treated with local treatment options. As no systemic therapy has been shown to improve overall survival for patients with metastatic uveal melanoma, there is no specific standard of care and patients should be directed to clinical trials. In the Netherlands, metastatic melanoma care has been centralized to 14 expert centers[36] improving management of metastatic melanoma patients, but also facilitating enrollment in clinical trials to get evidence-based treatment protocols. In our cohort in total 85 systemic therapies were

given in the context of a clinical trial, to 63 unique patients. The lack of availability of clinical trials was sometimes a reason to provide systemic therapy outside a clinical trial setting. These systemic therapies were registered for treatment of metastatic cutaneous melanoma and given to patients with metastatic uveal melanoma. In the present situation, decision making on available treatment options in metastatic UM patients occurs mainly on clinical characteristics leading to selection of patients for treatment in- and outside a clinical trial. The limited efficacy of checkpoint inhibitors in uveal melanoma has led to the agreement among members of the Dutch Working Group on immunotherapy and oncology (WIN-O) not to treat patients with immune checkpoint inhibitors outside a clinical trial. Combination studies on ipilimumab/nivolumab and novel immune-based approaches might be more promising[37].

In our cohort of UM patients, classic risk factors associated with survival, as elevated LDH and the presence of liver metastases[7,8] are confirmed to be negatively associated with survival (Figure 7). The distribution of metastases (Figure 2) in our cohort is consistent with data from the large Collaborative Ocular Melanoma Study trials[38].

Our observational cohort may suffer from limitations in terms of the registration of real-world data, sometimes leading to missing variables which might affect results, especially in smaller treatment groups. For instance, in the group of patients receiving local treatment (39 patients) information on WHO performance score was missing in 14 patients (35.9%). Another registration flaw was detected in the documentation of the molecular analysis, reporting a GNAQ and GNA11 mutation in 6.4% of the analyzed patients. These mutations are mutually exclusive. Other limitations relate to the choice of data to collect in a registry. From a scientific perspective, a broad set of clinical and pathological characteristics (including molecular and genomic alterations), treatment strategies, adverse events and survival is desirable. This is, however, not always feasible, and ongoing developments are more difficult to incorporate. At this time, the DMTR contains limited data on molecular and genomic tumor alterations.

Important strengths of our observational cohort are the complete overview of patient and metastatic tumor characteristics and treatment options available in the Netherlands between 2012 and 2018 for metastatic uveal melanoma patients. Differences in metastatic UM patients are most probably caused by differences in baseline characteristics and patient selection for specific treatment. However, this overview might be used by other authors for comparing survival between treatment groups and the impact of their treatment strategy applied.

Conclusions

We present baseline characteristics, mutation analysis and treatment strategies with the corresponding one-year survival of a nation-wide (full coverage) cohort of 175 patients with metastatic uveal melanoma in the Netherlands. Selection of patients for treatment was mainly based on clinical characteristics, showing elevated LDH (HR 9.0, 95% CI 5.63-14.35), and the presence of liver metastases (HR 2.09, 95% CI. 1.07-4.08) was negatively associated with survival in metastatic UM. The analysis of our observational cohort reflects the treatment choices made by physicians in Dutch melanoma expert centers. Our overview might be used by other authors for comparing survival between treatment groups and the impact of treatment strategy applied.

Supplementary Materials

The following are available online at http://www.mdpi.com/2072-6694/11/7/1007/s1, Figure S1: Kaplan-Meier Estimates per treatment strategy and level of LDH (normal LDH < 250 U/L vs. elevated LDH > 250 U/L).

Author Contributions

Conceptualization, A.J., M.K.v.d.K., M.F., M.G.S., M.J.A., A.C.v.A., F.W.P.J.v.d.B., A.J.M.v.d.E., M.G.F., J.W.B.d.G., J.B.A.G.H., G.A.P.H., R.H.K., W.H.J.K., M.L., D.P., R.S.v.R., K.P.M.S., A.J.t.T., G.V., M.W.J.M.W., M.C.T.v.Z., K.J.M.v.d.H. and E.K.; Formal analysis, A.J., M.K.v.d.K., M.F., M.W.J.M.W., K.J.M.v.d.H. and E.K.; Methodology, A.J., M.K.v.d.K., M.F., M.W.J.M.W., K.J.M.v.d.H. and E.K.; Visualization, A.J. and M.K.v.d.K.; Writing—original draft, A.J. and M.K.v.d.K.; Writing—review & editing, M.F., M.G.S., M.J.A., A.C.v.A., F.W.P.J.v.d.B., C.U.B., A.J.M.v.d.E., M.G.F., J.W.B.d.G., J.B.A.G.H., G.A.P.H., R.H.K., W.H.J.S.v.R., K.P.M.S., A.J.t.T., G.V., M.W.J.M.W., M.C.T.v.Z., K.J.M.v.d.H. and E.K.

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Conflicts of Interest

In relation to their contribution to this specific manuscript the authors declare no conflict of interest. Some authors however have consulting/advisory relationships and have received funding for other research projects. A.J. has in the past received travel/ accommodation expenses from Roche BV Netherlands.

A.C.v.A. has consulting/advisory relationships with Amgen, BMS, Novartis, MSD and Merck-Pfizer. He has received research funding from Amgen and Novartis and travel, accommodations, and expenses from Amgen and Novartis. C.U.B. has consulting/ advisory relationships with BMS, MSD, Roche, Novartis, GSK, Lilly and Pfizer. He has received research funding from Novartis. A.J.M.v.d.E. has consulting/advisory relationships with BMS, Roche, MSD and Novartis. He has received a study grant from R. MargreetG. Franken has received grants from Roche BV Netherlands, Daiichi Sanky, AbbVie, Pamgene and Gilead Sciences. Jan Willem B. de Groot has advisory relationships with BMS, MSD and Roche. J.B.A.G. Haanen has provided consultation, attended advisory boards, and/or provided lectures for MSD, BMS, Roche and Novartis for which NKI received honoraria. His institution has received grant support from BMS and Novartis. G.A.P.H. has consulting/advisory relationships with Roche, MSD, BMS and Novartis. Her institution has received research funding from BMS. Rutger H. Koornstra has received speaker fees from BMS, MSD and Roche. He has advisory relationships with BMS, MSD, Novartis and Roche and has received research grants from BMS and Roche. K.P.M.S. has consulting/advisory relationships with BMS and MSD and has received honoraria from Novartis, Roche and Pierre Fabre (paid to institution). E.K. has consulting/advisory relationships with BMS, Novartis, Roche, Amgen, Pierre Fabre (honoraria paid to institution) and has received research grants from BMS, Novartis.

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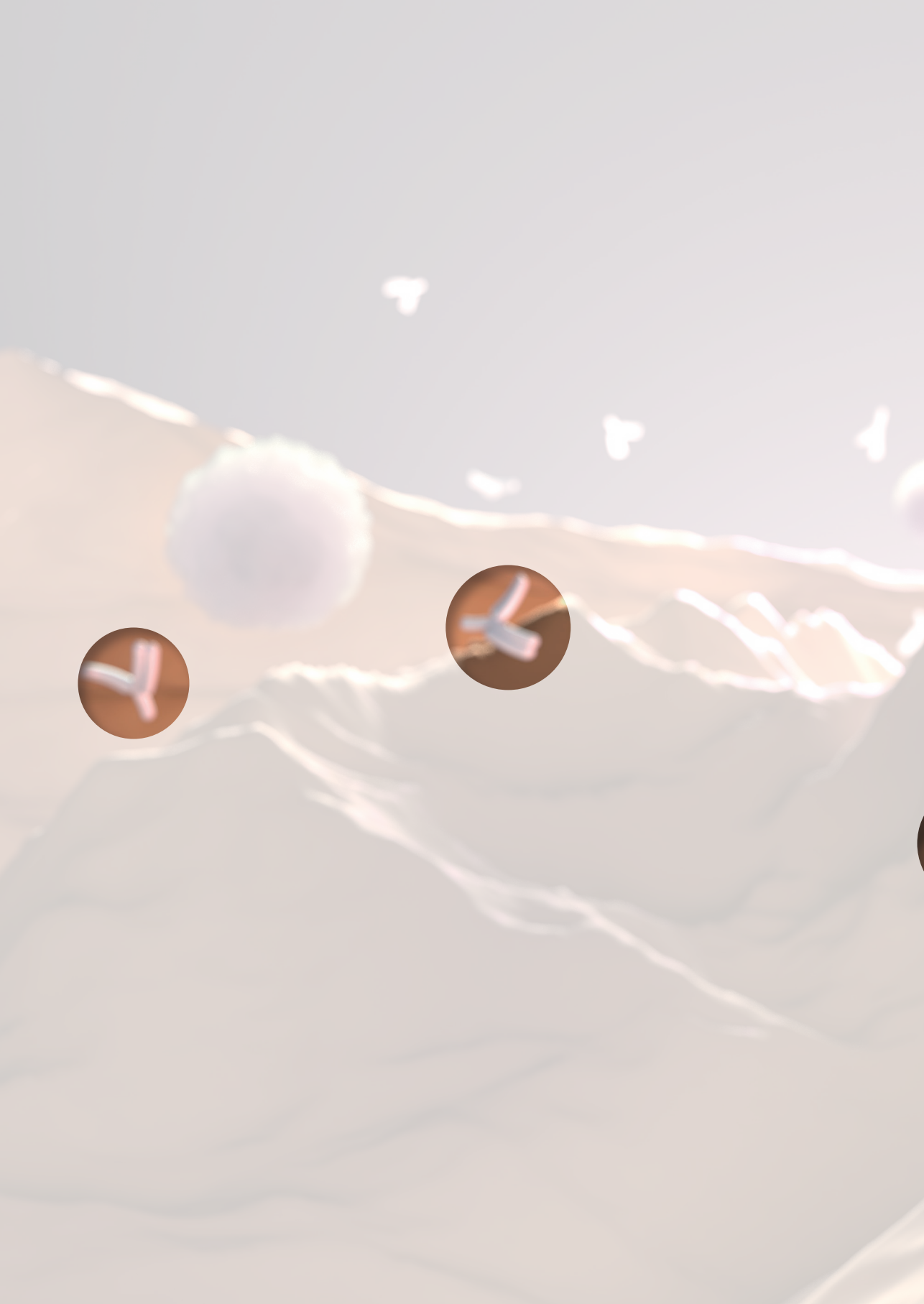
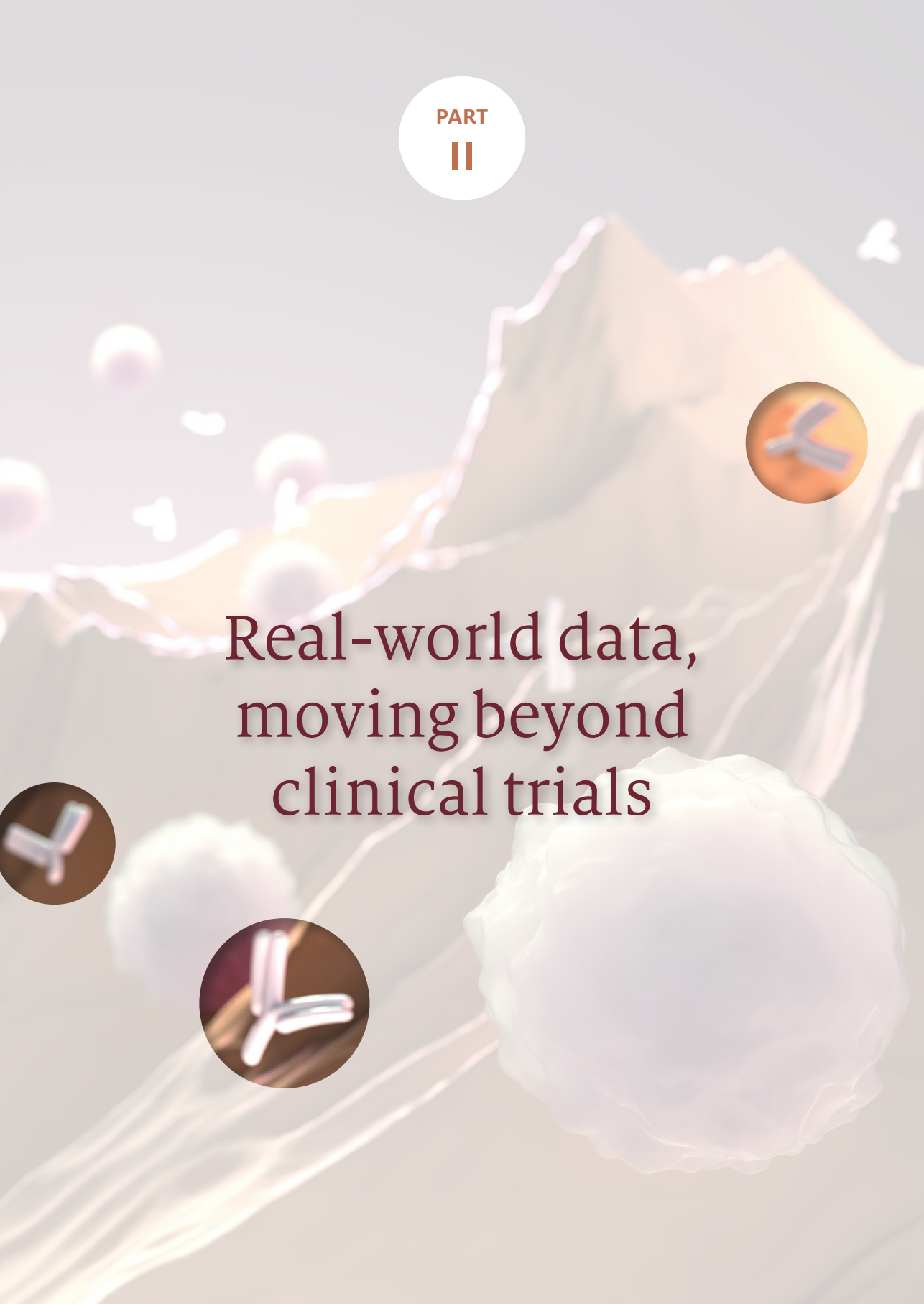


PART
II

Real-world data, moving beyond clinical trials



CHAPTER
5

Safety and efficacy of checkpoint inhibition in patients with melanoma and preexisting autoimmune disease: A Cohort Study

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Monique K. van der Kooij, Karijn P.M. Suijkerbuijk, Maureen J.B. Aarts, Franchette W.P.J. van den Berkmortel, Christian U. Blank, Marye J. Boers-Sonderen, Jesper van Breeschoten, Alfonsus J.M. van den Eertwegh, Jan Willem B. de Groot, John B.A.G. Haanen, Geke A.P. Hospers, Djura Piersma, Rozemarijn S. van Rijn, Albert J. ten Tije, Astrid A.M. van der Veldt, Gerard Vreugdenhil, Michiel C.T. van Zeijl, Michel W.J.M Wouters, Olaf M. Dekkers, Ellen Kapiteijn

Abstract

Background: Because immune checkpoint inhibition (ICI) can cause immune-related adverse events (irAEs) mimicking immunologic diseases, patients with preexisting autoimmune disease (AID) have been excluded from clinical trials.

Objective: To evaluate the safety and efficacy of ICI in patients with advanced melanoma with and without AID.

Design: Nationwide cohort study.

Setting: The Netherlands.

Patients: 4367 patients with advanced melanoma enrolled in the Dutch Melanoma Treatment Registry (DMTR) between July 2013 and July 2018 and followed through February 2019.

Measurements: Patient, clinical, and treatment characteristics; irAEs of grade 3 or higher; treatment response; and survival.

Results: A total of 415 patients (9.5%) had AID, categorized as rheumatologic AID (*n* = 227), endocrine AID (*n* = 143), inflammatory bowel disease (IBD) (*n* = 55), or "other" (*n* = 8). Of these, 228 patients (55%) were treated with ICI (vs. 2546 [58%] without AID); 87 were treated with anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4), 187 with anti-programmed cell death 1 (PD-1), and 34 with the combination. The incidences of irAEs of grade 3 or higher in patients with AID were 30% (95% CI, 21% to 41%) with anti-CTLA-4, 17% (CI, 12% to 23%) with anti-PD-1, and 44% (CI, 27% to 62%) with combination therapy; for patients without AID, the incidences were 30% (CI, 27% to 33%) (n = 916), 13% (CI, 12% to 15%) (*n* = 1540), and 48% (CI, 43% to 53%) (*n* = 388), respectively. Patients with AID more often discontinued anti-PD-1 treatment because of toxicity than patients without AID (17% [CI, 12% to 23%] vs. 9% [CI, 8% to 11%]). Patients with IBD were more prone to anti-PD-1-induced colitis (6/31 = 19% [CI, 7% to 37%]) than patients with other AIDs (3% [CI, 0% to 6%]) and patients without AID (2% [CI, 2% to 3%]).

The objective response rate was similar in patients with versus without AID who were treated with anti-CTLA-4 (10% [CI, 5% to 19%] vs. 16% [CI, 14% to 19%]), anti-PD-1 (40% [CI, 33% to 47%] vs. 44% [CI, 41% to 46%]), or the combination (39% [CI, 20% to 59%] vs. 43% [CI, 38% to 49%]). Survival did not differ between patients with and those without AID (median, 13 months [CI, 10 to 16 months] vs. 14 months [CI, 13 to 15 months]).

Limitation: Information was limited on AID severity and immunosuppressive treatment.

Conclusion: Response to ICI with anti-CTLA-4, anti-PD-1, or their combination for advanced melanoma and overall incidence of any irAEs of grade 3 or higher were similar in patients with and without preexisting AID. However, severe colitis and toxicity requiring early discontinuation of treatment occurred more frequently among patients with preexisting IBD, warranting close follow-up.

Primary Funding Source: The Netherlands Organization for Health Research and Development.

Introduction

Immune checkpoint inhibition (ICI) has greatly improved survival of patients with advanced (that is, unresectable stage III or IV) melanoma[1-6]. Both anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and anti-programmed cell death 1 (PD-1) have been approved by the U.S. Food and Drug Administration and the European Medicines Agency for the treatment of melanoma. The number of indications is rapidly expanding to other solid and hematologic tumors, so more patients with cancer will potentially benefit from these therapies.

Immune checkpoint inhibition can lead to long-lasting responses. However, its use can be hampered by serious immune-related adverse events (irAEs) that mimic classic autoimmune diseases (AIDs)[7]. Trials studying ICI have excluded patients with preexisting AIDs because of concerns about unleashing their underlying autoimmunity. Case reports typically describe unique manifestations and are not generalizable to the population at large, which has limited recently published reviews[8-10]. Recent retrospective studies concluded that patients with melanoma or non-small cell lung cancer and a preexisting AID had relatively frequent irAEs, although mild and easily manageable[11,12]. A recent article described the safety of anti-CTLA-4 and anti-PD-1 monotherapy for patients with inflammatory bowel disease (IBD); the authors concluded that treatment was associated with a higher rate of gastrointestinal AEs[13]. The aforementioned studies used retrospectively collected data with associated risk of bias, such as selection bias. Our current study used prospectively collected data from a nationwide registry. Our objective was to test the hypothesis that irAEs of grade 3 or higher occur more frequently in patients with advanced melanoma and AID than in patients without AID. Furthermore, we compared baseline characteristics, treatment choices, response, and survival after ICI.

Methods

Patients

Since July 2013, all patients with advanced melanoma in the Netherlands have been referred to 1 of 14 expert hospitals, and their data are prospectively registered in the Dutch Melanoma Treatment Registry (DMTR)[14]. Data are collected from patient files by trained data managers and approved by the treating physician. All patients diagnosed with unresectable stage III or IV melanoma in the Netherlands between July 2013 and July 2018 were included in our study. The data cutoff was February 2019; patients who stopped ICI before February 2019 were also included. All patients who were registered by their treating physician as having concomitant AID based on their medical history were compared with all other patients. Registered AIDs were IBD, endocrine AID

(hypo- or hyperthyroidism or Graves disease), rheumatoid AID (rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), scleroderma, sarcoidosis, or vasculitis), or "other" (all AIDs not listed here). The DMTR does not collect specific information on whether patients have type 1 or 2 diabetes. Given the age distribution in our study, we assumed that most of our patients would have type 2 diabetes. Therefore, patients who were registered as having diabetes and an AID were classified as "other" because further information on their exact AID was missing.

At baseline, the following immunosuppressive therapies were registered: corticosteroids, azathioprine, interferon, or "other" (including biologics). Anticancer treatment included ICI with anti-CTLA-4 (ipilimumab), anti-PD-1 (nivolumab or pembrolizumab), or their combination (nivolumab and ipilimumab) and targeted therapy with BRAF inhibitors (vemurafenib, dabrafenib, or encorafenib) and/or MEK inhibitors (cobimetinib, trametinib, or binimetinib). The DMTR contains information on patient and tumor characteristics, treatment regimens, AEs and irAEs of grade 3 or higher, and clinical outcome.

In compliance with Dutch regulations, use of DMTR data for research was approved by the Medical Ethics Review Committee of Leiden University Medical Center and was not considered subject to the Medical Research Involving Human Subjects Act.

Outcomes

The primary outcome of our study was the safety of ICI in patients with and without AID. The DMTR reports only treatment-related AEs of grade 3 or higher (according to the Common Terminology Criteria for Adverse Events, version 4.0). Toxicity related to ICI is considered to result from the drugs' immunologic activity and hence is called an irAE. Additional information on the clinical consequences of any grade of toxicity of the different systemic treatments was obtained from the variable "reason to stop treatment." Response evaluation in this uncontrolled, real-world setting is based on clinical judgment of the treating physician, in line with the RECIST (Response Evaluation Criteria in Solid Tumors) 1.1 criteria[15]. Responses were defined as follows: complete response was disappearance of all lesions, partial response was at least 30% decrease from baseline, progressive disease was at least 20% increase, and stable disease was neither partial response nor progressive disease.

Best overall response was the best response evaluation that a patient received after initiation of treatment until start of a new melanoma therapy or last follow-up visit. Objective response rate was defined as having complete or partial response.

Overall survival (OS) was calculated from date of diagnosis of advanced melanoma to date of last follow-up visit (censored observation) or date of death. Melanoma-specific survival (MSS) was calculated from date of diagnosis to date of melanoma-related death, date of last follow-up visit (censored observation), or other cause of death (censored observation). In a competing-risk model, non-melanoma-related death was considered a competing event. Progression-free survival (PFS) was calculated from start of systemic treatment until date of first progression according to the response evaluation or death.

Statistical Analysis

All patients who were included in the DMTR were also included in the analysis of baseline characteristics. Descriptive statistics were used to summarize baseline characteristics at diagnosis of advanced melanoma and start of treatment.

We did a Pearson χ^2 analysis to test whether immunosuppressive treatment in the presence of AID influenced choice of systemic treatment. To compare the safety of ICI between patients with and those without AID, all patients were included who received at least 1 infusion of anti-CTLA-4 or anti-PD-1. Patients who received sequential treatment with anti-PD-1 and anti-CTLA-4 were included in these analyses. Data on toxicity were coupled to the appropriate ICI by the trained data manager and treating physician. The 95% CIs of the proportions of patients with irAEs and of patients who had to stop ICI because of toxicity were compared in patients with versus without AID and in patients with AID who used versus did not use immunosuppressive treatment. All patients who received at least 1 response evaluation were included in the response evaluation, which was mainly based on the RECIST 1.1 criteria. However, some patients did not receive radiologic assessment because quickly progressing disease was clinically evident; these patients are registered as having progressive disease. Patients who had not yet been evaluated for response were not included in the analysis. Pearson χ^2 analyses were used to compare the objective response rate after ICI in patients with versus without AID.

For all patients in the DMTR, at least 1 visit was registered before data cutoff. Therefore, all patients could be included in the survival analysis. Kaplan-Meier estimates of OS, MSS, and PFS were calculated; the incidence of death was plotted for OS and MSS. We report both unadjusted and adjusted associations between AID and survival (OS, MSS, and PFS) with a Cox proportional hazards model. In addition, to estimate the melanoma-related mortality risk, a cumulative incidence competing-risk method was used. To estimate subdistribution hazard ratios and corresponding 95% CIs, Fine and Gray competing-risk models were used with melanoma-related death as event and

non-melanoma-related death as competing risk[16, 17]. We adjusted for the following prognostic factors: lactate dehydrogenase levels, Eastern Cooperative Oncology Group performance status, distant metastasis in at least 3 organ sites, brain metastases, *BRAF* mutation, and age. The proportional hazards assumption was checked by visual inspection.

We used SPSS, version 25.0 (IBM), to generate descriptive statistics; to perform Pearson χ^2 analysis, survival analysis according to the Kaplan-Meier method, and Cox regression; and to calculate risk estimates.

We used Stata, version 14.1 (StataCorp), to calculate the cause-specific cumulative incidence function in the presence of competing risk (non-melanoma-related death) by using the user-written stcompet command. The stcrreg command was used to implement the Fine and Gray approach. To plot the cumulative incidence functions, the stcurve command was used.

Figures were created in GraphPad Prism, version 8.1.1 (GraphPad Software).

Role of the Funding Source

Representatives of the pharmaceutical companies that sponsor the DMTR and The Netherlands Organization for Health Research and Development had no role in writing the manuscript, collecting or analyzing the data, or interpreting the results.

Results

Baseline Characteristics

Our nationwide cohort included 4367 patients with advanced melanoma. Four hundred fifteen patients (9.5%) had preexisting AID (Table 1). Appendix Table 1 shows numbers of patients with and without AID per hospital.

At diagnosis, patients with AID were older than those without AID (67 vs. 63 years), were more frequently female (53% vs. 41%), had higher Eastern Cooperative Oncology Group performance status, and more often used immunosuppressive medication (36% vs. 18%). Although patients with AID had melanoma metastases in fewer organs and less often had brain metastases, lactate dehydrogenase levels did not differ (Table 1). Appendix Table 2 shows the number of patients included per condition that was classified as AID.

TABLE 1 Baseline Characteristics at Diagnosis and Initial Melanoma Therapy in Patients with and without Autoimmune Disease*

Characteristics	AID (*n*=415)	No AID (*n*=3952)
Age at diagnosis		
Mean (range), y	66.5 (24-92)	62.7 (2-97)
<65 y	162 (39)	1999 (51)
≥65 y	253 (61)	1953 (49)
Sex		
Male	193 (47)	2345 (59)
Female	222 (53)	1607 (41)
ECOG performance status		
0	163 (39)	1845 (47)
1	120 (29)	1107 (28)
2,3 or 4	64 (15)	500 (13)
Unknown	68 (16)	499 (12)
LDH		
Normal	232 (56)	2266 (57)
250-500 U/l	89 (21)	845 (21)
>500 U/l	65 (16)	507 (13)
Missing	29 (7)	334 (9)
Metastasis in ≥3 organ sites		
Yes	113 (27)	1262 (32)
No	302 (73)	2690 (68)
Brain metastases		
Yes	87 (21)	1048 (27)
Symptomatic	62 (15)	706 (18)
No	272 (66)	2550 (64)
Unknown	56 (13)	354 (9)
Mutational profile		
BRAF mutation	181 (44)	1945 (49)
V600E	140 (34)	1481 (38)
V600K	21 (5)	241 (6)
NRAS mutation	78 (19)	721 (18)
No BRAF/NRAS mutation	156 (38)	1295 (33)
Immunosuppressive treatment		
Yes	148 (36)	699 (18)
Corticosteroids	121 (35)	686 (17)
Azathioprine	6 (2)	2
Interferon	0	1
Other	31 (9)	19 (1)
No	267 (64)	3253 (82)

Initial treatment		
Systemic	186 (45)	1850 (47)
Local & Systemic	97 (23)	949 (24)
Local	71 (17)	686 (17)
Other treatment	0	21 (1)
No treatment	61 (15)	446 (11)

AID=autoimmune disease, ECOG=Eastern Cooperative Oncology Group, LDH=Lactate dehydrogenase, ULN=upper limit of normal.
*Values are numbers (percentages) unless otherwise indicated. Percentages may not sum to 100 due to rounding.

Treatment Patterns

First-line treatment was systemic therapy in 68% of patients with AID and 71% of patients without. Figure 1 shows the cumulative number of first-line treatments with targeted therapy or ICI over time for patients with versus without AID. Systemic treatment choices were similar over time. Patients with AID receiving immunosuppressive treatment received first-line targeted therapy more frequently and ICI less frequently than patients with AID without immunosuppression (Figure 1).

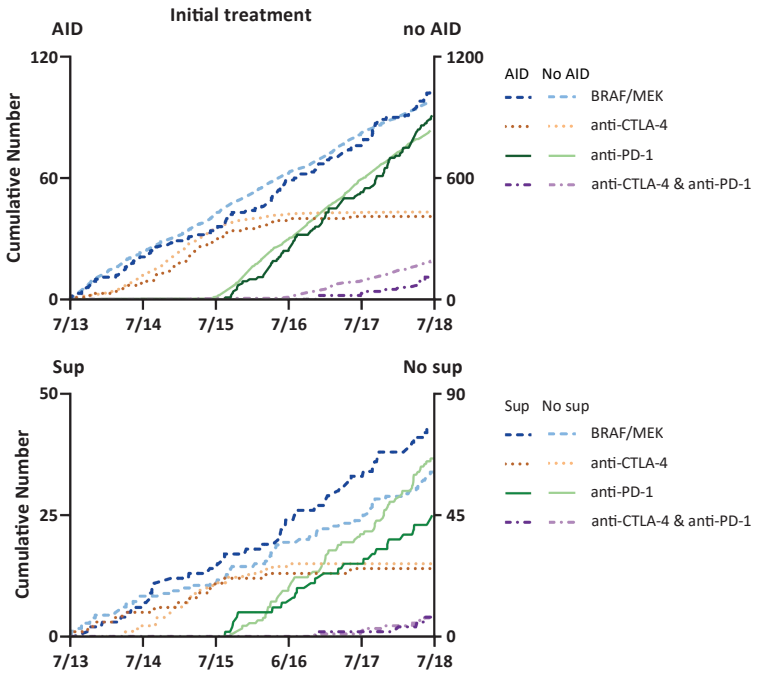

FIGURE 1 First-line systemic treatment initiated for advanced melanoma in patients with and without AID. AID = autoimmune disease; CTLA-4 = cytotoxic T lymphocyte–associated protein 4; PD-1 = programmed cell death 1; sup = immunosuppressive treatment. **Top**. Cumulative number of patients with and without AID treated with targeted therapy and immune checkpoint inhibition (ICI) over time since July 2013. **Bottom**. Cumulative number of patients with AID using sup and patients with AID not using sup receiving first-line targeted therapy and ICI since July 2013.

Timing of anti-CTLA-4 and anti-PD-1 treatment initiation was similar in patients with and without AID; almost half of the treated patients received these as first-line treatment (Appendix Table 3). Median follow-up time for patients with and without AID was 18 months for both with anti-CTLA-4; 14 and 15 months, respectively, after anti-PD-1 treatment initiation; and 3 and 5 months, respectively, after start of combination therapy with anti-CTLA-4 and anti-PD-1.

Choices for initial systemic treatment were similar among patients with IBD (*n* = 55), AID of endocrine origin (*n* = 143), and AID of rheumatologic origin (*n* = 227). Between 32% and 34% of patients in these groups did not receive systemic treatment; BRAF or MEK inhibition was prescribed to 24% to 26% of patients, anti-PD-1 to 20% to 24% of patients, and combination treatment with anti-CTLA-4 and anti-PD-1 to a minority of 2% to 3%. It seemed that patients with IBD received anti-CTLA-4 less often (6% [95% CI, 1% to 15%]) than those with rheumatologic (10% [CI, 7% to 15%]) or endocrine (12% [CI, 7% to 18%]) AID. However, the number of patients was limited.

Comparing second-line systemic treatment between patients with and those without AID, anti-CTLA-4 was less frequently prescribed to those with AID, whereas second-line treatment with anti-PD-1 tended to be prescribed more often, and targeted therapy prescription was similar.

Selection for ICI

Regardless of treatment line, 55% of patients with AID received ICI, versus 58% of patients without AID. When comparing patients with AID who received ICI (n = 143), targeted therapy (n = 104), another therapy (n = 107), and no initial treatment (n = 61), those receiving ICI more often had a normal level of lactate dehydrogenase before the start of treatment (71% [CI, 62% to 78%], 40% [CI, 31% to 50%], 10% [CI, 5% to 18%], and 21% [CI, 12% to 34%], respectively) (Appendix Table 4).

Anti-CTLA-4

Eighty-seven patients (21%) with AID were treated with anti-CTLA-4. Of these, 6 had IBD, 41 had a rheumatologic AID (2 vasculitis; 2 sarcoidosis; and 37 RA, SLE, or scleroderma), 43 had an endocrine AID (1 Graves disease and 42 hypo- or hyperthyroidism), and 2 had another AID.

Anti-PD-1

In 187 patients (42%) with AID, anti-PD-1 treatment was initiated; 31 had IBD, 89 had AID of rheumatologic origin (2 vasculitis; 3 sarcoidosis; and 84 RA, SLE, or scleroderma), 73 had AID of endocrine origin (all hypo- or hyperthyroidism), and 3 had AID of another origin.

Anti-CTLA-4 and Anti-PD-1

Thirty-four patients (8%) were treated with the combination of ipilimumab and nivolumab; 6 had IBD, 14 had AID of rheumatologic origin (3 sarcoidosis and 11 RA, SLE, or scleroderma), and 14 had AID of endocrine origin (all hypo- or hyperthyroidism).

TABLE 2 Number of Patients with Grade III/IV Immune-Related Adverse Events and Patients who Discontinued Therapy because of Toxicity.

	AID, n/N (% [95%CI])			no AID, n/N (% [95%CI])		
Immunosuppressive medication at baseline	**Yes**	**No**	**Total**	**Yes**	**No**	**Total**
Grade 3 or 4 irAEs						
Anti-CTLA-4	6/28 (21 [8-41])	20/59 (34 [22-47])	26/87 (30 [21-41])	24/104 (23 [15-32])	248/812 (31 [27-34])	272/916 (30 [27-33])
Anti-PD-1	10/68 (15 [7-25])	21/119 (18 [11-26])	31/187 (17 [12-23])	31/220 (14 [10-19])	175/1320 (13 [11-15])	206/1540 (13 [12-15])
Combination*	11/21 (52 [30-74])	4/13 (31 [9-61])	15/34 (44 [27-62])	38/83 (46 [35-57])	149/305 (49 [43-55])	187/388 (48 [43-53])
Treatment discontinued because of toxicity						
Anti-CTLA-4	2/28 (7 [1-24])	14/59 (24 [14-37])	16/87 (18 [11-28])	11/104 (11 [5-18])	127/812 (16 [13-18])	138/916 (15 [13-18])
Anti-PD-1	6/68 (9 [3-18])	25/119 (21 [14-29])	31/187 (17 [12-23])	20/220 (9 [6-14])	124/1320 (9 [8-11])	144/1540 (9 [8-11])
Combination*	2/13 (15 [2-45])	8/21 (38 [18-62])	10/34 (29 [15-47])	30/83 (36 [26-47])	115/305 (38 [32-43])	145/388 (37 [33-42])

AID = autoimmune disease; CTLA-4 = cytotoxic T lymphocyte–associated protein 4; irAE = immune-related adverse event; PD-1 = programmed cell death 1.
* Anti–CTLA-4 and anti–PD-1.

Safety of ICI

Anti-CTLA-4

The incidence of irAEs of grade 3 or higher associated with anti-CTLA-4 was 30% for both patients with and those without AID (Table 2; Appendix Table 5). No patients with AID died of toxicity, versus 3 patients without AID (0.3%).

Of the 28 patients who were receiving immunosuppressive treatment, 21% (CI, 8% to 41%) developed irAEs of grade 3 or higher, versus 34% (CI, 22% to 47%) of the 59 patients without. Because of the limited number of patients with AID treated with anti-CTLA-4, we could not draw any definite conclusions on the differences in reasons to terminate treatment or the influence of immunosuppressive treatment on toxicity.

Anti-PD-1

Incidence of irAEs of grade 3 or higher was similar in patients with and without AID (17% [CI, 12% to 23%] and 13% [CI, 12% to 15%], respectively) (Table 2; Appendix Table 6). No patients with AID died of toxicity, versus 5 patients without AID (0.3%).

Toxicity led to discontinuation of treatment more frequently in patients with AID (17% [CI, 12% to 23%]) than in those without (9% [CI, 8% to 11%]). Furthermore, patients with AID developed more colitis of grade 3 or higher (5% [CI, 3% to 10%] vs. 2% [CI, 2% to 3%]) (Appendix Table 6). The incidence of irAEs of grade 3 or higher did not differ in patients with AID who used versus did not use immunosuppressive treatment at baseline (15% [CI, 7% to 25%] of 68 patients vs. 18% [CI, 11% to 26%] of 119 patients, respectively) (Table 2).

Anti-CTLA-4 and Anti-PD-1

After combination therapy, 44% (CI, 27% to 62%) of 34 patients with versus 48% (CI, 43% to 53%) of 388 patients without AID had irAEs of grade 3 or higher (Table 2; Appendix Table 7). No patients with AID died of toxicity, versus 1 patient without AID (0.3%).

Specific AID Categories

Patients with IBD were more prone to anti-PD-1-induced colitis (6/31 = 19% [CI, 7% to 37%]) than those with other AIDs (3% [CI, 0% to 6%]) and those without AID (2% [CI, 2% to 3%]). In 5 of 6 patients with IBD who developed colitis, treatment with corticosteroids was initiated; 2 received additional treatment with tumor necrosis factor-a inhibitors, and 1 had an intestinal perforation. Because of the limited number of patients with IBD treated with anti-CTLA-4 with or without anti-PD-1, we could not draw any definite conclusions on the differences in safety between AID categories.

Response After ICI

Both best overall response and objective response rate after ICI were similar in patients with and without AID. The objective response rate after anti-CTLA-4 treatment was 10% (CI, 5% to 19%) of 78 patients with AID, versus 16% (CI, 14% to 19%) of 843 patients without AID. After anti-PD-1 treatment, 40% (CI, 33% to 47%) of 178 patients with AID had a response, versus 44% (CI, 41% to 46%) of 1491 patients without AID. Of 26 patients with AID treated with combination therapy, 39% (CI, 20% to 59%) had an objective response, versus 43% (CI, 38% to 49%) of 334 patients without AID (Appendix Table 8).

Survival

All Patients

Overall survival since diagnosis of advanced melanoma did not differ in patients with versus without AID (median, 13 months [CI, 10 to 16 months] vs. 14 months [CI, 13 to 15 months], respectively). Furthermore, there was no difference in crude or adjusted hazard ratios for MSS, OS, or PFS after ICI between patients with and those without AID (Figure 2; Appendix Table 9).

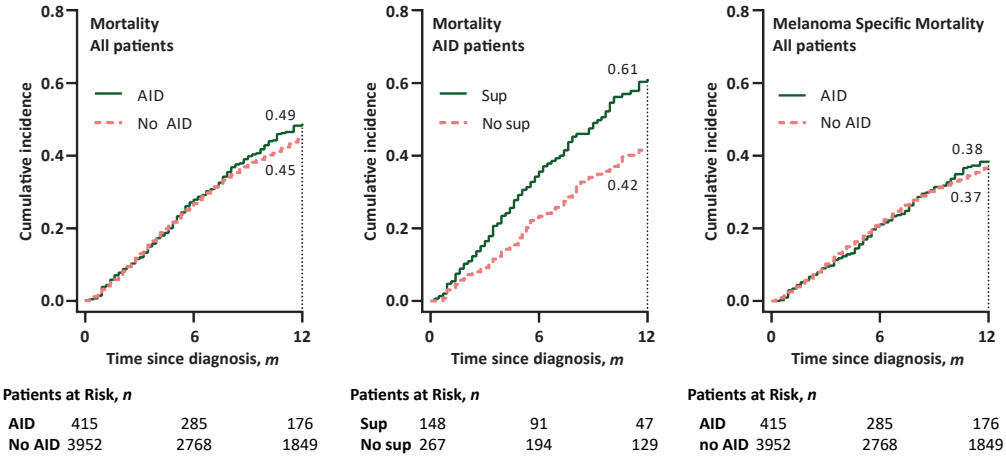

FIGURE 2 Cumulative incidence of mortality and melanoma-specific mortality.
AID = autoimmune disease; sup = immunosuppressive treatment. **Left**. Cumulative incidence of mortality of all patients with and without AID. **Center**. Cumulative incidence of mortality of patients with AID who use or do not use sup at baseline. **Right**. Cumulative incidence of melanoma-specific mortality of patients with and without AID.

Patients with AID who used immunosuppressive treatment at baseline seemed to have a higher cumulative incidence of death than patients with AID who did not use immunosuppressive treatment (Figure 2). However, this difference was no longer present after adjustment for known prognostic factors (adjusted hazard ratio, 1.18 [CI, 0.90 to 1.54]) (Appendix Table 10). The incidence of death was similar between AID categories (Appendix Figure).

Anti-CTLA-4

Overall survival was similar in patients with and without AID (median, 12 months [CI, 8 to 16 months] and 12 months [CI, 11 to 13 months], respectively). It did not differ between the 28 patients with AID who used immunosuppressive medication and the 59 patients with AID who did not (median, 10 months [CI, 8 to 12 months] and 16 months [CI, 7 to 25 months], respectively).

Anti-PD-1

Patients with and without AID had similar OS from start of anti-PD-1 therapy (median, 22 months [CI, 19 to 25 months] and 20 months [CI, 15 to 25 months], respectively). There was no statistically significant difference in OS between patients with AID with (n = 148) and without (n = 267) concomitant use of immunosuppressive treatment at baseline (median, 13 months [CI, 9 to 17 months] and 23 months [CI, 14 to 32 months], respectively).

Discussion

In the largest cohort reported to date, we observed that patients with AID and advanced melanoma in the Netherlands are treated with ICI as often as patients without AID. In patients with AID who used concomitant immunosuppressive medication, physicians seemed more hesitant to start ICI and more frequently prescribed targeted therapy. Incidence of irAEs of grade 3 or higher did not differ between patients with and those without AID. Toxicity and efficacy rates in patients with AID were largely in line with data from large phase 3 studies. Compared with anti-CTLA-4 monotherapy, anti-PD-1 with or without anti-CTLA-4 led to higher response rates and longer survival in both patients with and those without AID[3-6,18].

Half of the patients with advanced melanoma who are evaluated for ICI are not represented in phase 3 registration trials[19,20]. Patients with AID were excluded from these trials. To our knowledge, this is the first study to bridge this knowledge gap by presenting "real-world" data on the safety and efficacy of ICI on a national scale. In our population-based cohort, 9.5% of all patients with advanced melanoma had preexisting AID. This is higher than the estimated 7.6% to 9.4% described in nononcologic studies and national registry data[21].

Our findings on irAEs of grade 3 or higher after anti-CTLA-4 treatment in 87 patients with AID are in accordance with those of a previously published retrospective study by Johnson and colleagues[22], who described 30 patients with AID (incidence, 30% (CI, 21% to 41%) in our study vs. 33% (CI, 17% to 53%) in Johnson and colleagues').

The percentage of irAEs of grade 3 or higher after anti-PD-1 treatment in our patients with AID is similar to what Danlos and colleagues[23] reported. The difference in overall toxicity could be explained by the fact that Danlos and colleagues included grade 2 AEs in their analysis. The increased rate of treatment discontinuation due to toxicity in patients with AID in our study suggests that grade 2 irAEs might have been more frequent in our cohort as well[23]. A recent study using the DMTR database showed that patients who had toxicity management with tumor necrosis factor-α inhibitors had lower survival than those who were managed with steroids only[24]. In our study,

upfront use of immunosuppressive treatment was not clearly related to occurrence of irAEs of grade 3 or higher in patients with AID. The limited number of patients and events could explain why this difference was no longer statistically significant in multivariable analysis for patients with AID.

We compared treatment patterns in patients with different categories of AID. Patients with IBD were less often treated with anti-CTLA-4 than those with a rheumatologic or endocrine AID or those without AID. We speculate that this could be because of the known higher incidence of (gastrointestinal) AEs after this type of ICI or possibly fear of a flare of the preexisting IBD. The percentage of grade 3 or 4 colitis after anti-PD-1 treatment in our 31 patients with IBD was similar to that among the 85 patients in Abu-Sbeih and colleagues' retrospective study[13] (16% (CI, 7% to 37%) in our study vs. 19% (CI, 11% to 29%) in Abu-Sbeih and colleagues').

It was previously reported that the incidence of AEs after anti-PD-1 therapy differs among cancer types: Patients with melanoma have fewer AEs than those with, among others, ovarian cancer, sarcoma, or colorectal carcinoma[25]. A recent meta-analysis[26] compared the relative risk for AEs after anti-CTLA-4, anti-PD-1, and anti-programmed cell death ligand-1 treatment in multiple solid organ tumors compared with standard of care chemotherapy. Its subgroup analysis found similar odds ratios regardless of cancer type[26]. The similarities in relative risk strengthen our belief that our findings on safety of ICI in patients with advanced melanoma and AID might also be translatable to patients with other solid tumors.

A strength of our approach is that we used nation-wide, population-based data from the DMTR. All data are prospectively registered by trained data managers and approved by the treating physician. However, some limitations exist. Because only irAEs of grade 3 or higher are registered, mild to moderate flares of AID are not included in our analysis. Moreover, detailed information on exact type of AID, reason to prescribe immunosuppressive treatment, and prescribed dose is not available. The data presented reflect real-world treatment of patients with AID of rheumatologic or endocrine origin or IBD, but these data might not be generalizable to all AIDs. Rarer AIDs will be underrepresented in our cohort. Especially for myositis, myasthenia gravis, and Guillain-Barré syndrome, which are associated with high fatality rates when occurring as irAEs[27], caution is needed.

In 2017, combination therapy with anti-PD-1 and anti-CTLA-4 became readily available for patients with advanced melanoma in the Netherlands. Therefore, the number of patients treated with this combination is limited in our current database. It would be interesting to reevaluate the safety and efficacy of this combination therapy in patients with AID in the coming years.

In conclusion, we show that tumor response to ICI treatment with anti-CTLA-4, anti-PD-1, or their combination for advanced melanoma and incidence of irAEs of grade 3 or higher were similar in patients with and without preexisting AID of rheumatologic or endocrine origin in daily clinical practice. Therefore, we encourage physicians not to withhold ICI in most common AIDs. However, close monitoring in patients with IBD is advised because the incidence of severe colitis and early discontinuation of treatment due to toxicity was higher in this group.

Disclaimer

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Disclosures

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Author Contributions

Conception and design: M.K. van der Kooij, K.P.M. Suijkerbuijk, O.M. Dekkers, E. Kapiteijn. Analysis and interpretation of the data: M.K. van der Kooij, K.P.M. Suijkerbuijk, O.M. Dekkers, E. Kapiteijn. Drafting of the article: M.K. van der Kooij, K.P.M. Suijkerbuijk, O.M. Dekkers, E. Kapiteijn. Critical revision of the article for important intellectual content: M.K. van der Kooij, K.P.M. Suijkerbuijk, M.J.B. Aarts, F.W.P.J. van den Berkmortel, C.U. Blank, M.J. Boers-Sonderen, A.J.M. van den Eertwegh, J.W.B. de Groot, J.B.A.G. Haanen, G.A.P. Hospers, D. Piersma, R.S. van Rijn, A.J. ten Tije, A.A.M. van der Veldt, G. Vreugdenhil, M.C.T. van Zeijl, M.W.J.M Wouters, O.M. Dekkers, E. Kapiteijn. Final approval of the article: M.K. van der Kooij, K.P.M. Suijkerbuijk, M.J.B. Aarts, F.W.P.J. van den Berkmortel, C.U. Blank, M.J. Boers-Sonderen, J. van Breeschoten, A.J.M. van den Eertwegh, J.W.B. de Groot, J.B.A.G. Haanen, G.A.P. Hospers, D. Piersma, R.S. van Rijn, A.J. ten Tije, A.A.M. van der Veldt, G. Vreugdenhil, M.C.T. van Zeijl, M.W.J.M Wouters, O.M. Dekkers, E. Kapiteijn.

Provision of study materials or patients: K.P.M. Suijkerbuijk, M.J.B. Aarts, F.W.P.J. van den Berkmortel, C.U. Blank, M.J. Boers-Sonderen, A.J.M. van den Eertwegh, J.W.B. de Groot, J.B.A.G. Haanen, G.A.P. Hospers, D. Piersma, R.S. van Rijn, A.J. ten Tije, A.A.M. van der Veldt, G. Vreugdenhil, M.W.J.M Wouters, E. Kapiteijn. Statistical expertise: M.K. van der Kooij, O.M. Dekkers, E. Kapiteijn. Collection and assembly of data: M.K. van der Kooij, K.P.M. Suijkerbuijk, M.J.B. Aarts, F.W.P.J. van den Berkmortel, C.U. Blank, M.J. Boers-Sonderen, A.J.M. van den Eertwegh, J.W.B. de Groot, J.B.A.G. Haanen, G.A.P. Hospers, D. Piersma, R.S. van Rijn, A.J. ten Tije, A.A.M. van der Veldt, G. Vreugdenhil, M.W.J.M Wouters, E. Kapiteijn.

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Appendix

APPENDIX TABLE 1 Number of Included Patients With and Without Autoimmune Disease per Melanoma Treatment Center

Treatment Center	AID (*n* = 415), *n* (%)	No AID (*n* = 3952), *n* (%)	Total (*n* = 4367), *n*
1	11 (8.3)	122 (91.7)	133
2	22 (13.1)	146 (86.9)	168
3	7 (5.5)	120 (94.5)	127
4	22 (9.4)	213 (90.6)	235
5	29 (7.3)	368 (92.7)	397
6	77 (7.3)	971 (92.7)	1048
7	28 (13.1)	184 (86.8)	212
8	64 (12.5)	450 (87.5)	514
9	23 (9.3)	225 (90.7)	248
10	10 (9.6)	94 (90.4)	104
11	47 (12.0)	345 (88.0)	392
12	22 (12.6)	153 (87.4)	175
13	33 (7.9)	387 (92.1)	420
14	20 (10.3)	174 (89.7)	194

AID = autoimmune disease.

APPENDIX TABLE 2 Number of Patients Included per Condition Classified as AID*

AID Category	Subtype	Patients, *n*
IBD	IBD	55
Endocrine	Hypo-/hyperthyroidism	141
Endocrine	Graves disease	3
Rheumatoid	RA/SLE/scleroderma	213
Rheumatoid	Sarcoidosis	10
Rheumatoid	Vasculitis	5
Other	Other	8

AID = autoimmune disease; IBD = inflammatory bowel disease; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus.

* Twenty patients with AID had multiple AIDs: 5 had rheumatoid and IBD, 12 had rheumatoid and endocrine, 1 had IBD and AID of endocrine origin, 1 had both Graves disease and hypo-/hyperthyroidism, and 1 had RA/SLE/scleroderma and sarcoidosis.

APPENDIX TABLE 3 Treatment Episodes Where Immune Checkpoint Inhibition Was Initially Given in Patients With and Without Autoimmune Disease*

Treatment Episode†	Anti–CTLA-4		Anti–PD-1		Anti–CTLA-4 and Anti–PD-1	
	AID (*n* = 87)	No AID (*n* = 916)	AID (*n* = 187)	No AID (*n* = 1540)	AID (*n* = 14)	No AID (*n* = 108)
1	41 (47)	432 (47)	91 (49)	834 (54)	1 (7)	38 (35)
2	30 (33)	372 (40)	59 (32)	456 (30)	8 (57)	50 (46)
3	10 (12)	80 (9)	27 (14)	159 (10)	3 (21)	12 (11)
4	4 (5)	25 (3)	7 (4)	70 (4)	1 (7)	4 (5)
5	1 (1)	6 (1)	2 (1)	10 (1)	0	2 (2)
≥6	1 (1)	1	1	11 (1)	1 (7)	1 (1)

AID = autoimmune disease; CTLA-4 = cytotoxic T lymphocyte–associated protein 4; PD-1 = programmed cell death 1.

* Values are numbers (percentages).

† Identified as the line of treatment after diagnosis of advanced melanoma. The first episode in which a patient received each individual drug is shown.

APPENDIX TABLE 4 Baseline Characteristics of Patients With Autoimmune Disease at Time of Initial Antitumor Treatment*

Characteristic	ICI (n = 143)†	Targeted Therapy (n = 104)‡	Other Treatment (n = 107)§	No Treatment (n = 61)‖
Age at treatment decision				
Mean (range), y	67 (24–89)	63 (32–87)	65 (33–89)	74 (33–92)
<65 y	53 (37)	53 (51)	48 (45)	8 (13)
≥65 y	90 (63)	51 (49)	59 (55)	53 (87)
Time since registration				
Median (IQR), wk	5 (1–9)	4 (0–8)	7 (0–14)	–
Sex				
Male	62 (43)	51 (49)	50 (47)	30 (49)
Female	81 (57)	53 (51)	57 (53)	31 (51)
ECOG performance status				
0	73 (51)	31 (30)	46 (43)	13 (21)
1	47 (33)	39 (37)	21 (20)	13 (21)
2, 3, or 4	11 (8)	24 (23)	13 (12)	16 (26)
Unknown	12 (8)	10 (10)	27 (25)	19 (31)
Lactate dehydrogenase level				
Normal	101 (71)	42 (40)	11 (10)	13 (21)
4.17–8.33 μkat/L (<2x ULN)	32 (22)	29 (28)	66 (62)	23 (38)
>8.33 μkat/L (>2x ULN)	8 (6)	30 (29)	21 (20)	7 (11)
Missing	2 (1)	3 (3)	9 (8)	18 (30)
Metastasis in ≥3 organ sites				
Yes	36 (25)	45 (43)	18 (17)	14 (23)
No	107 (75)	59 (57)	89 (83)	47 (77)
Brain metastases				
Yes	24 (17)	29 (28)	26 (24)	8 (13)
Symptomatic	13 (9)	22 (21)	21 (20)	6 (10)
No	107 (75)	65 (62)	60 (56)	40 (66)
Unknown	12 (8)	10 (10)	21 (20)	13 (21)
Immunosuppressive treatment				
Yes	43 (30)	43 (41)	35 (33)	27 (44)
No	100 (70)	61 (59)	72 (67)	34 (56)

ECOG = Eastern Cooperative Oncology Group; ICI = immune checkpoint inhibition; IQR = interquartile range; ULN = upper limit of normal.

* Values are numbers (percentages) unless otherwise indicated.

† Anti–programmed cell death 1, anti–cytotoxic T lymphocyte–associated protein 4, or the combination.

‡ BRAF inhibitors and MEK inhibitors.

§ Dacarbazine, talimogene laherparepvec, surgery, radiation, radiofrequency ablation, or hyperthermia.

‖ Best supportive care.

APPENDIX TABLE 5 Number of Patients with Grade III/IV Immune-related Adverse Events, Therapy Discontinuation, and Adverse Events Consequences following Anti-CTLA-4 Treatment*

Variable	IBD AID *n*=6	Endo AID *n*=43	Rheum AID *n*=41	All AID *n*=87†	no AID *n*=916
Reason to stop treatment					
Pre-planned	1 (17)	25 (58)	22 (54)	47 (54)	536 (59)
Progression	2 (33)	5 (12)	10 (24)	16 (18)	165 (18)
Toxicity	3 (50)	8 (19)	6 (15)	16 (18)	138 (15)
Patient choice	-	-	-	-	3
Patient Condition	-	3 (7)	2 (5)	5 (6)	46 (5)
Death	-	1 (2)	-	1 (1)	13 (1)
Other	-	-	1 (2)	1 (1)	4
Unknown	-	-	-	-	2
Not applicable	-	1 (2)	-	1 (1)	9 (1)
Grade III-IV irAE	2 (33)	13 (30)	12 (29)	26 (30)	272 (30)
Colitis	2 (33)	7 (16)	8 (20)	16 (18)	137 (15)
Intestinal perforation	-	-	1 (2)	-	4
Hepatitis	-	3 (7)	-	3 (3)	23 (3)
Adrenal insufficiency	-	-	2 (4)	2 (2)	25 (3)
Myelotoxicity	-	-	1 (2)	1 (1)	7 (1)
Neuropathy	-	-	-	-	2
Hypophyses insufficiency	-	-	2 (5)	2 (2)	50 (6)
Thyroid insufficiency	-	-	2 (5)	2 (2)	21 (2)
Skin toxicity	-	3 (7)	1 (2)	3 (3)	21 (2)
Uveitis	-	-	-	-	2
Other	-	3 (7)	4 (10)	7 (8)	56 (6)
Toxicity consequences					
Immunosuppressive medication	2 (33)	12 (28)	11 (27)	24 (28)	258 (28)
Corticosteroids	2 (33)	12 (28)	9 (22)	22 (25)	221 (24)
TNFa blocker	-	-	-	-	-
Other	2 (33)	6 (14)	6 (15)	14 (16)	85 (9)
Admitted outpatient clinic	-	-	1 (2)	1 (1)	14 (2)
Admitted hospital	-	7 (16)	10 (24)	17 (20)	192 (21)
Permanent damage	-	-	-	-	9 (3)
Death due to toxicity	-	-	-	-	3

AE = adverse event; AID = autoimmune disease; CTLA-4 = cytotoxic T lymphocyte–associated protein 4; IBD = inflammatory bowel disease; irAE = immune-related AE.

* Values are numbers (percentages).

† Five patients had both an AID of endocrine and one of rheumatologic origin. Two patients had an AID classified as "other."

APPENDIX TABLE 6 Number of Patients with Grade III/IV Immune-related Adverse Events, Therapy Discontinuation, and Adverse Events Consequences following Anti-PD-1 Treatment*

Variable	IBD AID *n*=31	Endo AID *n*=73	Rheum AID *n*=89	All AID *n*=187†	no AID *n*=1540
Reason to stop treatment					
Pre-planned	2 (7)	13 (18)	6 (7)	21 (11)	227 (15)
Progression	18 (58)	32 (44)	42 (47)	89 (48)	744 (48)
Toxicity	6 (19)	12 (16)	15 (17)	31 (17)	145 (9)
Patient choice	-		1 (1)	1	25 (2)
Patient condition	-	3 (4)	7 (8)	10 (5)	64 (4)
Death	-	1 (1)	1 (1)	2 (1)	30 (2)
Other	-	5 (7)	2 (2)	7 (4)	71 (5)
Unknown	1 (3)	-	-	1	10
Not applicable	4 (13)	7 (10)	15 (17)	25 (13)	224 (15)
Grade III-IV irAE	7 (23)	13 (18)	12 (14)	31 (17)	206 (13)
Colitis	6 (19)	-	4 (5)	10 (5)	34 (2)
Intestinal perforation	1 (3)	-	1 (1)	2 (1)	17 (1)
Hepatitis	-	3 (4)	3 (3)	5 (3)	25 (2)
Decline in renal function	-	-	1 (1)	1	11
Nephritis	-	-	1 (1)	-	9 (1)
Dyspnea	-	1 (1)	-	1	5
Pneumonia	-	2 (3)	2 (2)	4 (2)	17 (1)
Adrenal insufficiency	-	1 (1)	-	1	11 (1)
Myelotoxicity	-	-	-	-	6
Neuropathy	-	-	-	-	5
Hypophyses insufficiency	-	-	-	-	8 (1)
Thyroid insufficiency	-	1 (1)	-	1 (1)	13 (1)
Fatigue	-	2 (3)	1 (1)	3 (2)	12 (1)
Rash	-	-	2 (2)	2	10 (1)
Pruritis	-	-	-	-	2
Vitiligo	-	1 (1)	-	1	6
Other	1 (3)	5 (7)	3 (3)	9 (5)	82 (5)
Toxicity consequences					
Immunosuppressive medication	7 (23)	11 (15)	11 (12)	28 (15)	177 (12)
Corticosteroids	6 (19)	10 (14)	8 (9)	23 (12)	141 (9)
TNFa blocker	2 (7)	1 (1)	2 (2)	5 (3)	15 (1)
Other	-	-	-	-	12 (1)
Admitted outpatient clinic	3 (10)	1 (1)	-	4 (2)	8 (1)
Admitted hospital	4 (13)	9 (12)	6 (7)	19 (10)	104 (7)
Permanent damage	2 (7)	-	1 (1)	3 (2)	10 (1)
Death due to toxicity	-	-	-	-	5

AE = adverse event; AID = autoimmune disease; IBD = inflammatory bowel disease; irAE = immune-related AE; PD-1 = programmed cell death 1.
* Values are numbers (percentages).
† Five patients had both an AID of endocrine and one of rheumatologic origin, and 4 patients had both IBD and an AID of rheumatologic origin. Three patients had an AID classified as "other."

APPENDIX TABLE 7 Number of Patients With Grade 3 or 4 irAEs, Therapy Discontinuation, and AE Consequences After Anti-CTLA-4 Plus Anti-PD-1 Combination Treatment*

Variable	IBD AID (*n* = 6)	AID of Endocrine Origin (*n* = 14)	AID of Rheumatologic Origin (*n* = 14)	All AID (*n* = 34)	No AID (*n* = 388)
Reason to stop treatment					
Preplanned	–	–	–	–	26 (7)
Progression	2 (33)	4 (29)	4 (29)	10 (29)	100 (26)
Toxicity	1 (17)	7 (50)	2 (14)	10 (29)	145 (37)
Patient choice	–	–	–	–	5 (1)
Patient condition	1 (17)	1 (7)	1 (7)	3 (9)	29 (8)
Death	–	–	–	–	22 (6)
Other	–	–	–	–	6 (2)
Unknown	–	1 (7)	–	1 (3)	2 (1)
Not applicable	2 (33)	1 (7)	7 (50)	10 (29)	53 (14)
Grade 3 or 4 irAE	1 (17)	9 (64)	5 (36)	15 (44)	187 (48)
Diarrhea	–	–	1 (7)	1 (3)	26 (7)
Colitis	–	3 (21)	2 (14)	5 (15)	61 (16)
Hepatitis	1 (17)	3 (21)	1 (7)	5 (15)	73 (19)
Nephritis	–	–	–	–	7 (2)
Pneumonia	–	–	–	–	14 (4)
Adrenal insufficiency	–	–	–	–	6 (2)
Myelotoxicity	–	1 (7)	–	1 (3)	2 (1)
Neuropathy	–	–	–	–	5 (1)
Pituitary insufficiency	1 (17)	1 (7)	–	2 (6)	18 (5)
Thyroid insufficiency	–	1 (7)	1 (7)	2 (6)	12 (3)
Fatigue	–	–	–	–	3 (1)
Rash	1 (17)	–	–	1 (3)	15 (4)
Pruritus	–	–	–	–	5 (1)
Vitiligo	–	–	–	–	1
Other	–	3 (21)	3 (21)	6 (18)	39 (10)
Toxicity consequences					
Immunosuppressive medication	1 (17)	8 (57)	5 (36)	14 (41)	178 (46)
Corticosteroids	1 (17)	8 (57)	4 (29)	13 (38)	165 (43)
Tumor necrosis factor-a blocker	–	2 (14)	1 (7)	3 (9)	36 (9)
Other	1 (17)	2 (14)	–	3 (9)	22 (6)
Admitted to outpatient clinic	–	–	–	–	9 (2)
Admitted to hospital	1 (17)	6 (43)	2 (14)	9 (27)	112 (29)
Permanent damage	–	–	–	–	5 (1)
Death due to toxicity	–	–	–	–	1

AE = adverse event; AID = autoimmune disease; CTLA-4 = cytotoxic T lymphocyte–associated protein 4; IBD = inflammatory bowel disease; irAE = immune-related AE; PD-1 = programmed cell death 1.
* Values are numbers (percentages).

APPENDIX TABLE 8 Tumor Response After Immune Checkpoint Inhibition in Patients With and Without AID*

Treatment and Response	AID	No AID
Anti–CTLA-4	***n* = 78**	***n* = 843**
PD	40 (51 [40–63])	437 (52 [48–55])
SD	30 (38 [28–50])	270 (32 [29–35])
PR	6 (8 [3–16])	87 (10 [8–13])
CR	2 (3 [0–9])	49 (6 [4–8])
ORR†	8 (10 [5–19])	136 (16 [14–19])
Anti–PD-1	***n* = 178**	***n* = 1491**
PD	63 (35 [28–43])	502 (34 [32–36])
SD	44 (25 [19–32])	337 (23 [21–25])
PR	50 (28 [22–35])	455 (30 [28–33])
CR	21 (12 [7–17])	197 (13 [12–15])
ORR†	71 (40 [33–47])	652 (44 [41–46])
Anti–CTLA-4 and anti–PD-1	***n* = 26**	***n* = 334**
PD	12 (46 [27–67])	133 (40 [35–45])
SD	4 (15 [4–35])	57 (17 [13–22])
PR	9 (35 [17–56])	115 (34 [29–40])
CR	1 (4 [0–20])	29 (9 [5–12])
ORR†	10 (39 [20–59])	144 (43 [38–49])

AID = autoimmune disease; CR = complete response; CTLA-4 = cyto- toxic T lymphocyte–associated protein 4; ORR = objective response rate; PD = progressive disease; PD-1 = programmed cell death 1; PR = partial response; SD = stable disease.

* Values are numbers (percentages [95% CIs]).

† PR + CR.

APPENDIX TABLE 9 OS, MSS, and PFS for Patients With and Without AID

Patient Group and Survival	Events, n/N		HR (95% CI)	Adjusted HR (95% CI)
	AID	No AID		
All patients				
OS	258/415	2431/3952	1.04 (0.92–1.19)	0.98 (0.86–1.11)
MSS*				
Cox proportional hazards model	183/415	1859/3952	0.97 (0.83–1.13)	0.93 (0.80–1.08)
Competing-risk model	183/415	1859/3952	0.94 (0.81–1.09)	0.95 (0.81–1.11)
By initial treatment				
Anti–CTLA-4				
OS	59/87	662/916	0.95 (0.73–1.24)	0.90 (0.69–1.18)
MSS*				
Cox proportional hazards model	44/87	532/916	0.88 (0.65–1.20)	0.85 (0.62–1.16)
Competing-risk model	44/87	532/916	0.72 (0.45–1.14)	0.68 (0.42–1.11)
PFS	76/87	813/916	0.99 (0.78–1.25)	0.95 (0.75–1.20)
Anti–PD-1				
OS	91/187	725/1540	1.14 (0.92–1.42)	1.08 (0.87–1.34)
MSS*				
Cox proportional hazards model	68/187	573/1540	1.08 (0.84–1.39)	1.03 (0.80–1.32)
Competing-risk model	68/187	573/1540	1.14 (0.78–1.70)	1.12 (0.76–1.66)
PFS	126/187	1025/1540	1.15 (0.96–1.38)	1.11 (0.92–1.34)
Anti–CTLA-4 and anti–PD-1				
OS	14/34	178/388	1.13 (0.66–1.95)	-
MSS*				
Cox proportional hazards model	13/34	160/388	1.17 (0.67–2.06)	-
Competing-risk model	13/34	160/388	0.83 (0.31–2.23)	-
PFS	19/34	244/388	1.16 (0.73–1.86)	-

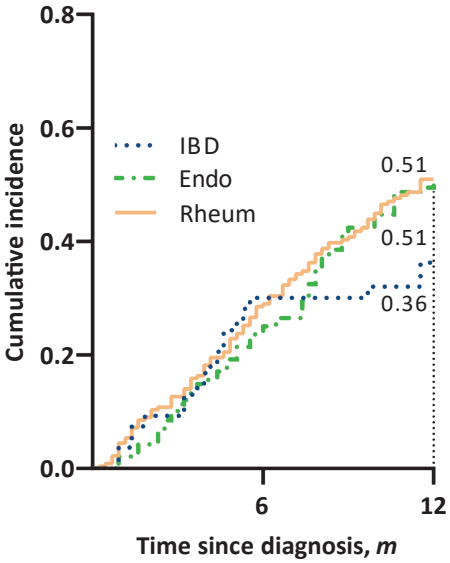
AID = autoimmune disease; CTLA-4 = cytotoxic T lymphocyte–associated protein 4; HR = hazard ratio; MSS = melanoma-specific survival; OS = overall survival; PD-1 = programmed cell death 1; PFS = progression-free survival.

* Calculated both using the Cox proportional hazards model and using the competing-risk model. In the competing-risk model, the subdistribution adjusted HR is shown.

APPENDIX TABLE 10 OS, MSS, and PFS for Patients with AID who use or do not use Immunosuppressive Medication.

Patient group and survival	Events, n/N		HR (95% CI)	Adjuste HR (95% CI)
	Immuno-suppressive medication	No Immuno-suppressive medication		
All patients				
OS	105/148	153/267	1.57 (1.23-2.02)	1.18 (0.90-1.54)
MSS*				
Cox proportional hazards model	73/148	110/267	1.15 (1.12-2.03)	1.02 (0.94-1.40)
Competing-risk model	73/148	110/267	1.33 (0.99-1.78)	0.87 (0.62-1.24)
Initial treatment				
Anti-CTLA-4				
OS	21/28	38/59	1.45 (0.85-2.47)	-
MSS*				
Cox proportional hazards model	17/28	27/59	1.65 (0.90-3.03)	-
Competing-risk model	17/28	27/59	1.29 (0.50-3.33)	-
PFS	24/28	52/59	1.22 (0.75-1.99)	-
Anti-PD-1				
OS	40/68	51/119	1.41 (0.93-2.14)	-
MSS*				
Cox proportional hazards model	33/68	35/119	1.69 (1.05-2.72)	-
Competing-risk model	33/68	35/119	2.34 (1.15-4.72)	-
PFS	50/68	76/119	1.22 (0.85-1.74)	-
Anti-CTLA-4 & anti-PD-1				
OS	7/13	7/21	1.27 (0.44-3.69)	-
MSS*				
Cox proportional hazards model	6/13	7/21	1.07 (0.35-3.24)	-
Competing-risk model	6/13	7/21	0.62 (0.06-5.92)	-
PFS	8/13	11/21	0.92 (0.36-2.31)	-

AID = autoimmune disease; CTLA-4 = cytotoxic T lymphocyte–associated protein 4; HR = hazard ratio; MSS = melanoma-specific survival; OS = overall survival; PD-1 = programmed cell death 1; PFS = progression-free survival.
* Calculated both using the Cox proportional hazards model and using the competing-risk model. In the competing-risk model, the subdistribution adjusted HR is shown.



Patients at Risk, *n

IBD	55	37	30
Endo	143	104	63
Rheum	227	150	86

APPENDIX FIGURE Cumulative incidence of mortality in patients with AID.

AID = autoimmune disease; IBD = inflammatory bowel disease.
* Some patients had multiple AIDs: 5 had rheumatoid AID and IBD, 12 had rheumatoid and endocrine AIDs, and 1 had IBD and endocrine AID.

CHAPTER
6

Age Does Matter in Adolescents and Young Adults versus Older Adults with Advanced Melanoma; A National Cohort Study Comparing Tumor Characteristics, Treatment Pattern, Toxicity and Response

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Monique K. van der Kooij, Marjolein J.A.L. Wetzels, Maureen J.B. Aarts, Franchette W.P.J. van den Berkmortel, Christian U. Blank, Marye J. Boers-Sonderen, Miranda P. Dierselhuis, Jan Willem B. de Groot, Geke A.P. Hospers, Djura Piersma, Rozemarijn S. van Rijn, Karijn P.M. Suijkerbuijk, Albert J. ten Tije, Astrid A.M. van der Veldt, Gerard Vreugdenhil, Michel W.J.M. Wouters, John B.A.G. Haanen, Alfonsus J.M. van den Eertwegh, Esther Bastiaannet, Ellen Kapiteijn

Abstract

Cutaneous melanoma is a common type of cancer in Adolescents and Young Adults (AYAs, 15-39 years of age). However, AYAs are underrepresented in clinical trials investigating new therapies and the outcomes from these therapies for AYAs are therefore unclear. Using prospectively collected nation-wide data from the Dutch Melanoma Treatment Registry (DMTR), we compared baseline characteristics, mutational profiles, treatment strategies, grade 3-4 adverse events (AEs), responses and outcomes in AYAs (n = 210) and older adults (n = 3775) who were diagnosed with advanced melanoma between July 2013 and July 2018. Compared to older adults, AYAs were more frequently female (51% versus 40%, p = 0.001), and had a better Eastern Cooperative Oncology Group performance status (ECOG 0 in 54% versus 45%, p = 0.004). BRAF and NRAS mutations were age dependent, with more BRAF V600 mutations in AYAs (68% versus 46%) and more NRAS mutations in older adults (13% versus 21%), $p < 0.001$. This finding translated in distinct first-line treatment patterns, where AYAs received more initial targeted therapy. Overall, grade 3-4 AE percentages following first-line systemic treatment were similar for AYAs and older adults; anti-PD-1 (7% versus 14%, p = 0.25), anti-CTLA-4 (16% versus 33%, p = 0.12), anti-PD-1 + anti-CTLA-4 (67% versus 56%, p = 0.34) and BRAF/MEK-inhibition (14% versus 23%, p = 0.06). Following anti-CTLA-4 treatment, no AYAs experienced a grade 3-4 colitis, while 17% of the older adults did (p = 0.046). There was no difference in response to treatment between AYAs and older adults. The longer overall survival observed in AYAs (hazard ratio (HR) 0.7; 95% CI 0.6-0.8) was explained by the increased cumulative incidence of non-melanoma related deaths in older adults (sub-distribution HR 2.8; 95% CI 1.5-4.9), calculated by competing risk analysis. The results of our national cohort study show that baseline characteristics and mutational profiles differ between AYAs and older adults with advanced melanoma, leading to different treatment choices made in daily practice. Once treatment is initiated, AYAs and older adults show similar tumor responses and melanoma-specific survival.

Introduction

Over the past decade, various systemic treatment options have become available for patients with advanced melanoma. These therapies include; antibodies targeting immune checkpoint T-lymphocyte-associated protein 4 (anti-CTLA-4), programmed cell death protein 1 (anti-PD-1) and targeted therapy against the BRAF kinases and MEK. In the Adolescent and Young Adult (AYA) population, defined as anyone between the ages of 15 and 39 years old, melanoma is less common when compared to adults older than 40 years of age. The 1-year incidence age-standardized risk for older adults in the Netherlands was 68.1 per 100,000 persons in 2018, when compared to 10.3 for AYAs. The same difference in 1-year incidence could be observed on a global scale; 8.7 per 100,000 persons in older adults versus 0.89 in AYAs[1]. Melanoma remains, however, one of the most frequently occurring cancers in AYAs, accounting for 4% of all cancers diagnosed in this age group[2].

Even though melanoma accounts for an important fraction of disease in AYAs, it is relatively uncommon in this age group when looking at the whole population. Therefore, only few patients are included in phase 3 studies. Current knowledge on prognostic factors and treatment strategies for (advanced) melanoma patients derives from these large phase 3 trials in patients with a median age varying between 53 and 62[3-8] years of age. The relevance of the results from these trials for AYAs is therefore unclear. Over the past years, multiple differences in melanoma characteristics between the AYAs and (older) adults have been suggested.

Daryanani et al. demonstrated that adolescents, defined as patients between 12 and 19 years of age, more often have locally advanced superficial spreading melanoma (SSM) as compared to adults. On the other hand, adults are more frequently diagnosed with nodular melanoma (NM)[9]. Indini et al. showed that melanoma was more common in male patients above 39 years of age, whereas females were more commonly afflicted with melanoma when under 39 years of age[10].

Mutations in genes encoding BRAF and NRAS proteins are the most common mutations found in melanoma, where approximately 50% of melanomas harbor a BRAF mutation and about 20% carry an NRAS mutation[11]. One of the most striking findings in smaller retrospective studies is that a higher incidence of BRAF mutations was observed in AYAs as compared to older adults[12], whereas NRAS mutations are more frequently detected in adults[13]. Differences in mutational profile and extent and manner of dissemination could influence the treatment choices made in daily clinical practice in AYAs versus older adults. Data on initiated treatments, efficacy and survival are currently lacking.

Using prospectively collected real-life nation-wide data from the Dutch Melanoma Treatment Registry (DMTR), we had a unique opportunity to study this specific group in a large dataset. Our aim was to validate the previously described differences and similarities between melanomas that are detected in AYAs versus older patients of ≥40 years of age. First, we compared baseline characteristics of AYAs and older adults. Second, we compared treatment strategies, treatment-related AEs and progression-free survival (PFS). Additionally, we performed a competing risk analysis for non-melanoma-related death, next to overall survival (OS) and disease-specific survival (DSS) analyses.

Materials and Methods

Data Source

Advanced melanoma patients in the Netherlands, irrespective of the type of primary melanoma, are registered in the DMTR following referral to one of the 14 expert hospitals in the Netherlands. The nation-wide centralization of advanced melanoma patients and their registration in the DMTR was initiated in 2012 to assure safety and quality of melanoma care[32]. Up till May 2018, patients between 15 and 18 years of age were mostly treated at the previously mentioned 14 expert hospitals. Since then, all children <18 years of age are referred to the department of Pediatric Oncology at the Princess Máxima Center. Information on patient and tumor characteristics, treatment regimens, grade 3-4 AEs (according to the Common Terminology Criteria for Adverse Events, version 4.0) and clinical outcomes have since been entered into the DMTR. Data are collected from patient files by trained data managers and approved by the treating physicians. The study was conducted in accordance with the Declaration of Helsinki. In compliance with Dutch regulations, the DMTR was approved by a medical ethical committee (METC Leiden University Medical Center, 2013) and is not considered subject to the Medical Research Involving Human Subjects Act.

Patients

Between July 2013 and July 2018, 4367 patients with advanced melanoma were registered in the DMTR, follow-up data cut-off was set at March 1st, 2019. The patients with missing data on gender ($n = 1$) and patients under the age of 15 years old ($n = 6$) were excluded from the analysis. Furthermore, patients with mucosal and uveal melanoma were excluded from analysis ($n = 375$). After including all eligible patients, 3985 patients were analyzed according to their age at registration of advanced melanoma. Treatment strategies were categorized as systemic therapy and non-systemic treatment. Systemic therapy was further subdivided into chemotherapy (Dacarbazine/DTIC), immune checkpoint inhibition (anti-CTLA-4, anti-PD-1 or a combination of both), targeted

therapy (BRAF-inhibition with or without MEK-inhibition) and "other" systemic therapy. Treatment strategies could have been initiated either as standard care or in the context of participation in a clinical trial. Non-systemic treatment was further subdivided into metastasectomy (systemic therapy "no", surgery "yes" and metastatic lesion identified) and radiotherapy (palliative yes versus no). Differences in treatment pattern between AYAs and older adults were assessed using a Pearson's chi-square test. In adherence with international guidelines and literature, AYAs were defined as all patients between 15 and 39 years of age[33].

Statistical Analysis

Patients were classified according to age group for all analyses; AYAs versus older adults. Patient baseline characteristics, type of primary tumor, localization of metastases (based on TNM 7th edition[34]), number of organ sites involved and frequencies of systemic therapy administration were determined using descriptive statistics. The difference between categorical variables for the different age groups was tested with a Pearson's chi-square test. A median test for independent medians tests was used to compare the time from primary diagnosis until advanced disease.

A univariable Cox analysis using the variables "gender" (male versus female), "ECOG performance status" (ECOG 0, ECOG 1 or ECOG 2), "LDH level" (not elevated, elevated within 2× upper limit of normal or strongly elevated >2× upper limit of normal), "brain metastases" (yes versus no), "distant metastasis in ≥3 organ sites" (yes versus no), "histologic type of melanoma" (superficial spreading versus nodular and versus other), "location primary tumor" (unknown primary, head-and-neck region, trunk, extremities or acral) and "BRAF mutation" (whether a BRAFV600E or BRAFV600K mutation was present versus absent) was performed. Subsequently, a multivariable Cox regression model was estimated, including the following prognostic factors; LDH level, ECOG performance status, distant metastasis in ≥3 organ sites, the presence of brain metastases, BRAF mutation[35-37]. The histologic subtype of primary melanoma (specifically superficial spreading and nodular) was not added to the multivariable analysis as the prognostic value in the advanced setting seems limited. A recent study showed no difference in survival between these subtypes following immune checkpoint inhibition. Nodular melanoma had a worse prognosis following targeted therapy. However, LDH level, ECOG performance status and number of metastases were not added in their multivariate analysis[38].

To compare the safety of initial systemic treatment between AYAs and older adults, all included patients received at least one infusion/treatment with anti-CTLA-4, anti-PD-1, anti-CTLA-4 and anti-PD-1 or BRAF/MEK inhibition. Differences in grade 3-4 toxicity were tested with a Pearson's chi-square test.

Response evaluation in this uncontrolled real-world setting was based on clinical judgement of the medical team and was partially based on the RECIST 1.1 criteria. The BOR is the best evaluation that a patient received after initiation of treatment, until the start of a new melanoma therapy, or last visit at the treating physician; CR, PR, SD or PD. The ORR is defined by clinicians as having CR and PR. Differences in ORR between AYAs and older adult patients was tested with a Pearson's chi-square test. Due to the limited number of AYAs in the different response groups, no test was performed to assess the possible statistical difference in BOR.

OS, PFS and DSS were used to estimate survival probabilities[39]. As it is known that younger patients have a longer life expectancy, we used the cumulative incidence competing risk method (CIRC) to estimate melanoma-related mortality risk. To estimate survival, cumulative incidence curves with non-melanoma-related death as competing risk were used. To estimate sHR and corresponding 95% CI, Fine and Gray competing risk models were used with melanoma-related death as event and non-melanoma-related death as competing risk. Follow-up started at first visit after the diagnosis of advanced disease[40,41].

All statistical analyses were conducted using SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. IBM Corp, Armonk, NY, USA) and STATA (StataCorp. 2015. Stata Statistical Software: Release 14.1. College Station, StataCorp LP, Lakeway Drive College Station, TX, USA).

Results

Patient Selection and Baseline Characteristics

Between July 2013 and July 2018, 4367 patients were registered in the DMTR database. In total, 3985 advanced melanoma patients were eligible for analysis; 210 between 15 and 39 years of age (AYAs) (5%) versus 3775 older adult patients (95%). For details on patient selection, see Figure 1.

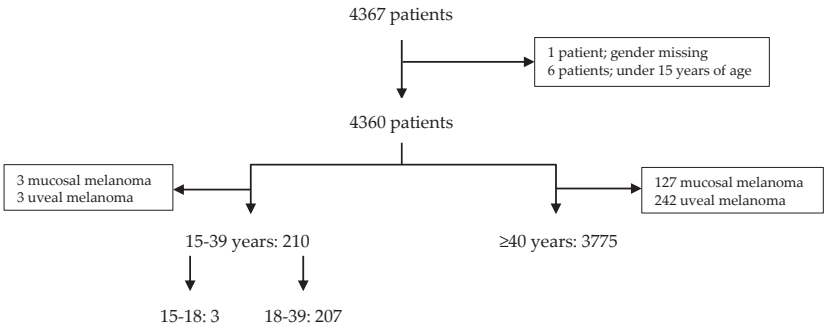

FIGURE 1 Patient selection for statistical analysis.

TABLE 1 Clinical and tumor characteristics of AYAs and older adult with advanced melanoma, and their primary melanomas. Eastern Cooperative Oncology Group (ECOG), lactate dehydrogenase (LDH), metastatic stage (M-stage).

Characteristic	AYA	Older adult	*p*-Value
Patients; *n*	210	3775	
Median age, year (range)	34 (15–39)	65 (40–97)	
Gender; *n* (%)			0.001
Male	102 (48.6)	2261 (59.9)	
Female	108 (51.4)	1514 (40.1)	
ECOG PS; *n* (%)			0.004
0	114 (54.3)	1694 (44.9)	
1	46 (21.9)	1090 (28.9)	
≥2	17 (8.1)	513 (13.6)	
Unknown	33 (15.7)	477 (12.6)	
LDH; *n* (%)			0.72
Normal	124 (59.0)	2171 (57.5)	
Elevated (<2xULN)	40 (19.0)	817 (21.6)	
High (≥2xULN)	30 (14.3)	472 (12.5)	
Unknown	16 (7.6)	315 (8.3)	
Metastasis in ≥3 organ sites; *n* (%)	68 (32.4)	1239 (32.8)	0.90
M-stage; *n* (%)			0.28
M1a	22 (10.5)	444 (11.8)	
M1b	15 (7.1)	403 (10.7)	
M1c	169 (80.5)	2829 (74.9)	
Unknown	4 (1.9)	99 (2.6)	
Brain metastasis; *n* (%)	60 (28.6)	1053 (27.9)	0.83
Symptomatic	42 (70.0)	715 (68.0)	0.74
Mutational profile; *n* (%)			<0.001
BRAF V600 mutation	143 (68.1)	1721 (45.6)	
BRAF V600E mutation	140 (66.7)	1466 (38.8)	
BRAF V600K mutation	3 (1.4)	255 (6.8)	
NRAS mutation	27 (12.9)	777 (20.6)	
No BRAF V600 or NRAS	40 (19.0)	1277 (33.8)	
Type of primary melanoma; *n* (%)			0.003
Superficial spreading	101 (48.1)	1535 (40.7)	
Nodular	26 (12.4)	832 (22.0)	
Other/unknown	83 (39.5)	1408 (37.3)	

Characteristic	AYA	Older adult	*p*-Value
Location primary melanoma; *n* (%)			0.003
Unknown primary	49 (23.3)	571 (15.1)	
Head and neck	38 (18.1)	525 (13.9)	
Trunk	64 (30.5)	1433 (38.0)	
Extremities	55 (26.2)	1142 (30.3)	
Acral	4 (1.9)	104 (2.8)	
Breslow thickness; *n* (%)			<0.001
≤2mm	90 (42.9)	1214 (32.2)	
2–4mm	43 (20.5)	943 (25.0)	
>4mm	20 (9.5)	754 (20.0)	
Unknown	57 (27.1)	864 (22.9)	

Differences in clinical and tumor characteristics between AYAs and older adults are shown in Table 1. Overall, AYAs were more frequently female, with a better ECOG performance status. Characteristics associated with tumor spread, including lactate dehydrogenase levels (LDH), Metastatic stage (M-stage) and the presence of brain metastases, were comparable between the two groups.

Characteristics of the primary melanoma are shown in the lower part of Table 1. AYAs had more SSM, while the primary was more frequently NM in older adults ($p = 0.003$). Furthermore, the primary tumor location was more often unknown in AYAs (23.3% versus 15.1%) or located in the head/neck region (18.1% versus 13.9%), while older adults had more primary melanomas on the trunk (38.0% versus 30.5%), $p = 0.003$. AYAs more often had thinner melanomas (Breslow thickness ≤2 mm) when compared to older adults, 42.9% versus 32.2%, $p < 0.001$.

Tumor Mutations

As shown in Table 1, AYAs more frequently harbor a BRAF V600 mutation (68.1% versus 45.6%), while older adults more frequently harbor an NRAS mutation (20.6% versus 12.9%) or have no BRAF V600 nor NRAS mutation (33.8% versus 19.0%), ($p < 0.001$). When further analyzing the percentage of patients having a BRAF V600 or NRAS mutation over different age groups, it was shown that the presence of a BRAF mutation is age dependent and shows a clear decrease of mutations over the 7 age groups in Figure 2. In the youngest AYA patient group between 15 and 29 years of age, 63.8% of patients harbored a BRAF V600 mutation, while of the patients of 80 years and older only 24.8% had this mutation.

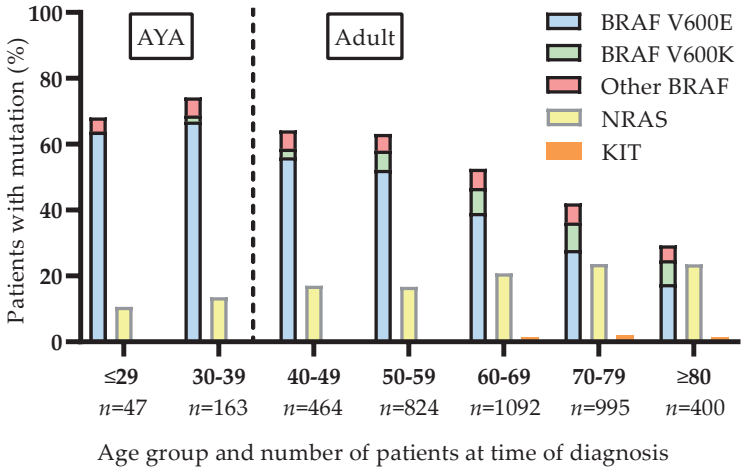

FIGURE 2 Incidence of BRAF, NRAS and KIT mutations in different age groups. Data on Adolescents and Young Adults (AYA) and older adults (Adult) is shown.

Furthermore, the percentage of patients with an NRAS mutation increased with age. In the youngest patient group, 10.6% harbored an NRAS mutation, in patients of 80 years and over this was 23.5%. Overall, the incidence of KIT mutations was low (1.2%). There seemed to be a slight increase over age, from 0% to 1.5% in the oldest patient group.

Initial Treatment

We analyzed the first-line treatment of all patients. Treatment patterns between AYAs and older adults differed significantly, $p < 0.001$. More AYAs were initially treated with BRAF/MEK-inhibition (35.2%) versus older adults (26.6%). Although the percentage of patients treated with immune checkpoint inhibitors did not differ between AYAs (33.8%) and older adults (37.6%), AYAs were given combination therapy with anti-PD-1 + anti-CTLA-4 more frequently (10.0% versus 4.5%), whereas monotherapy with anti-PD-1 was preferred in older adults (22.4% versus 14.8%), see Figure 3.

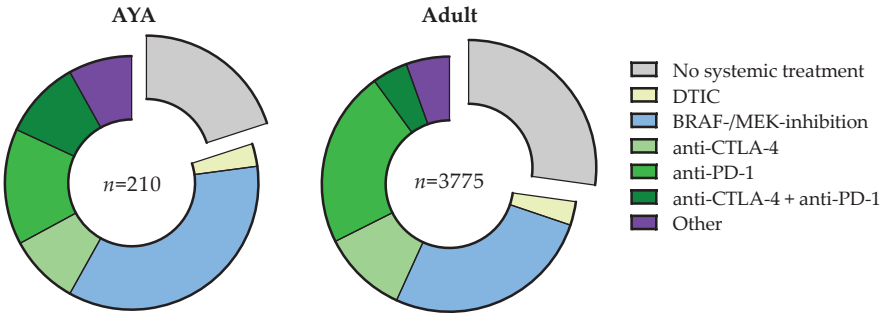

FIGURE 3 First-line melanoma treatment initiated in Adolescents and Young Adults (AYA) and older adults (Adult).

The initiation of BRAF/MEK-inhibition in AYAs remained constant over time since the start of our registry. Once anti-PD-1 was introduced, it largely replaced anti-CTLA-4 as first-line immune checkpoint inhibition in both AYAs and older adults. However, anti-PD-1 became more popular in older adults, while anti-CTLA-4 + anti-PD-1 was prescribed more to AYAs, see Figure S1.

In AYAs, 42 patients (20.0%) did not receive any systemic treatment first-line versus 1027 older adults (27.2%). Twenty-nine AYAs underwent metastasectomy as first-line treatment versus 385 of the adults (69.0% versus 37.5%, $p < 0.001$). This difference was no longer significant after stratification for ECOG performance status, LDH level or M-stage. There was no difference in the percentage of patients receiving radiotherapy (33.3% versus 38.2%, $p = 0.53$). This treatment was given in the palliative setting in 64.3% of the AYAs and 50.3% in older adults.

Treatment Toxicity

There was no difference in the occurrence of grade 3-4 AEs in AYAs or older adults for anti-PD-1 (6.5% versus 13.7%, $p = 0.25$), anti-CTLA-4 (15.8% versus 32.6%, $p = 0.12$) or anti-PD-1 with anti-CTLA-4 (66.7% versus 55.6%, $p = 0.34$), nor following treatment with BRAF/MEK inhibitors (13.5% versus 22.8%, $p = 0.06$), see Figure 4. Data on types of grade 3-4 AEs is provided in Table S1.

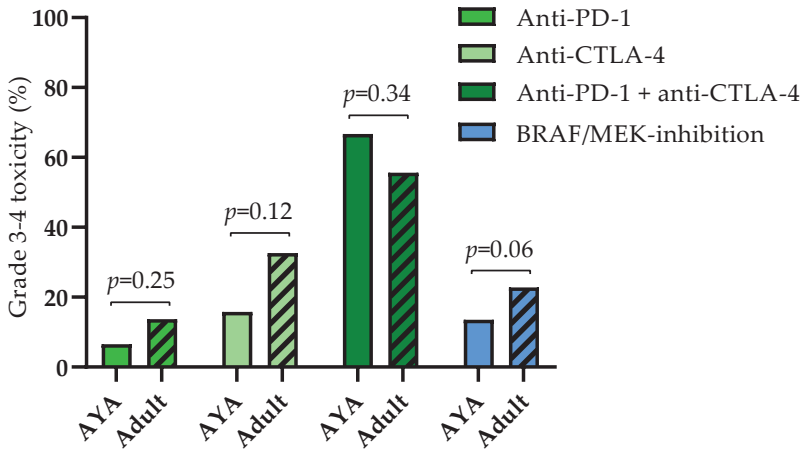

FIGURE 4 Toxicity rates following initial treatment for advanced melanoma of both Adolescents and Young Adults (AYAs) and older adults (Adult).

Although colitis is one of the most frequently reported AEs following anti-CTLA-4 treatment in large phase 3 trials, AYAs did not seem to be affected. Following initial

treatment with anti-CTLA-4 none of the 19 AYAs developed a grade 3-4 colitis, while 71 (17.4%) of the older adults did, $p = 0.046$. When we expanded the analysis, including all anti-CTLA-4 treated patients, regardless of the treatment line, we found that only 1 of the 48 (2.1%) AYAs experienced a grade 3-4 colitis. When we analyzed all older adults who received anti-CTLA-4 in any line of treatment, 134 of the 893 patients developed a 3-4 grade colitis (15.0%). Therefore, the difference in grade 3-4 colitis between AYAs and older adults remained significant, regardless of the timing of treatment, $p = 0.013$.

Furthermore, AYAs more often developed a grade 3-4 hepatitis following anti-PD-1 and anti-CTLA-4 combination treatment when compared to older adults (9 out of 21 versus 36 out of 169, $p = 0.03$). The relatively high incidence of hepatitis in AYAs was only seen in the first line of treatment. When we analyzed all patients that were ever treated with combination checkpoint inhibition 26.4% of AYAs and 18.0% of older adults developed a hepatitis ($p = 0.14$).

Response to Systemic Treatment

We compared the best overall response (BOR) and overall response rates (ORR) between AYAs and older adults following either initial anti-PD-1, anti-CTLA-4, anti-PD-1 + anti-CTLA-4 or BRAF/MEK-inhibition, see Table 2. Although none of the treatment groups showed a difference in ORR, there was a difference in BOR between AYAs and older adults treated with initial anti-PD-1. AYAs more often had a complete response (CR) (38.7% versus 16.7%), while older adults had more partial response (PR) (35.6% versus 16.1%).

TABLE 2 Best overall response and objective response rate following systemic treatment in Adolescents and Young Adults (AYAs) and older adults. Best overall response (BOR) was classified as either; progressive disease (PD), stable disease (SD), partial response (PR) or complete response (CR). Objective response rate (ORR) was the combination of PR and CR.

Anti-PD-1	**AYAs (*n* = 31)**	**Older adults (*n* = 779)**	**p-Value**
PD	9 (29.0)	196 (25.2)	
SD	5 (16.1)	176 (22.6)	
PR	5 (16.1)	277 (35.6)	
CR	12 (38.7)	130 (16.7)	
ORR	17 (54.8)	407 (52.2)	0.78
Anti-CTLA-4	**AYAs (*n* = 17)**	**Older adults (*n* = 385)**	
PD	10 (58.8)	166 (43.1)	
SD	5 (29.4)	143 (37.1)	
PR	1 (5.9)	45 (11.7)	
CR	1 (5.9)	31 (8.1)	
ORR	2 (11.8)	76 (19.7)	0.42

Anti-PD-1 and anti-CTLA-4	AYAs (*n* = 18)	Older adults (*n* = 146)	
PD	8 (44.4)	35 (24.0)	
SD	0	28 (19.2)	
PR	7 (38.9)	69 (47.3)	
CR	3 (16.7)	14 (9.6)	
ORR	10 (55.6)	83 (56.8)	0.92
BRAF/MEK inhibitor	**AYAs (*n* = 68)**	**Older adults (*n* = 923)**	
PD	15 (22.1)	148 (16.0)	
SD	12 (17.6)	272 (29.5)	
PR	36 (52.9)	452 (49.0)	
CR	5 (7.4)	51 (5.5)	
ORR	41 (60.3)	503 (54.5)	0.35

Survival

There was no difference in PFS following systemic therapy in AYAs and older adults, see Figure 5. The 1-year PFS for anti-PD-1 was 42.2% (95% CI 23.8-60.6) in AYAs and 44.1% (95% CI 40.6-47.6) in older adults, $p = 0.93$. Following anti-CTLA-4 treatment, 1-year PFS of AYAs was 15.8% (95% CI 0-32.3) and 16.7% (95% CI 12.9-20.4) for older adults, $p = 0.51$. The combination treatment of anti-PD-1 and anti-CTLA-4 yielded a 1-year PFS in AYAs of 50.0% (95% CI 28.0-72.0) and 40.2% (95% CI 32.0-48.4) in older adults, $p = 0.60$.

There were also no differences in PFS following targeted therapy: 1-year PFS in AYAs was 38.8% (95% CI 27.2-50.4), and 35.0% (95% CI 31.9-38.1) in older adults, $p = 0.58$. In Figure 5, both the crude HR for progression and adjusted HR are shown.

There was an OS advantage of AYAs over older adults, with a 1-year survival of 64.7% (95% CI 58.0-71.4) versus 55.0% (95% CI 53.4-56.6), $p < 0.001$. The HR for OS in AYAs was 0.69 (95% CI 0.57-0.84, $p < 0.001$) as compared to older adults (Table 3). The OS benefit of AYAs was higher in patients without a BRAF V600 mutation. However, even after adjusting for known prognostic factors (including BRAF V600 mutation) the influence of age group remained significant; adjHR 0.68 (95% CI 0.56-0.83, $p < 0.001$).

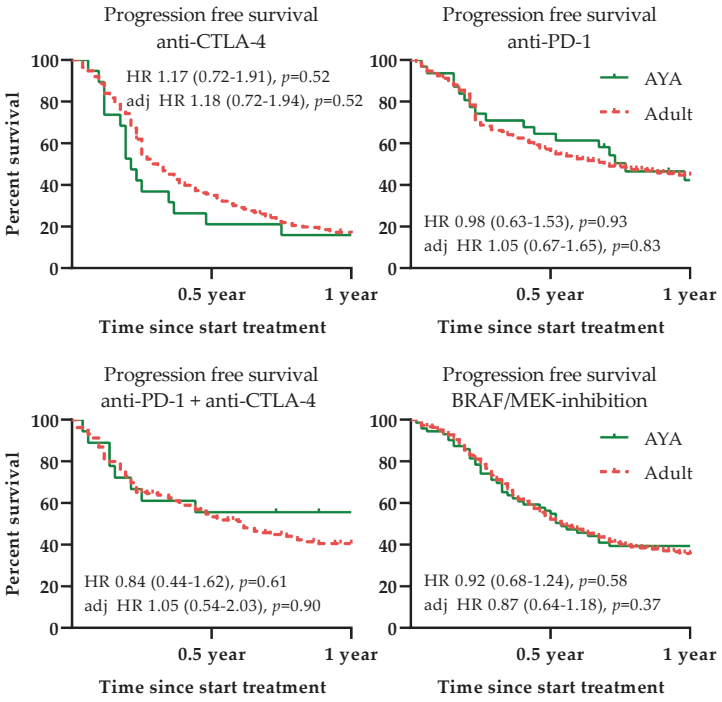

FIGURE 5 Progression free survival following first-line systemic therapy in Adolescents and Young Adults (AYAs) and older adult (Adult) patients. Hazard ratio (HR) for progression is provided, along with the adjusted HR (adjusted for: lactate dehydrogenase level, Eastern Cooperative Oncology Group performance status, distant metastasis in ≥3 organ sites, the presence of brain metastases and the presence of a BRAF V600 mutation).

TABLE 3 Overall survival, disease specific survival and competing risk analyses of all advanced melanoma patients.

	Events (*n*)		Crude HR			Adjusted HR		
	AYA	**Adult**	**HR**	**95% CI**	***p*-Value**	**HR**	**95% CI**	***p*-Value**
OS	102	2292	0.69	0.57–0.84	<0.001	0.68	0.56–0.83	<0.001
DSS	90	1728	0.81	0.66–1.00	0.06	0.79	0.64–0.98	0.03
Competing Risk	90	1728	0.90*	0.73–1.11	0.32	0.92*	0.75–1.13	0.43
Non-Melanoma								
nMSS	12	574	0.32	0.18–0.57	<0.001	0.33	0.18–0.58	<0.001
Competing risk	12	574	0.36*	0.20–0.63	<0.001	0.37*	0.21–0.67	<0.001

Data on Cox proportional hazard model for overall survival (OS), disease specific survival (DSS), non-melanoma specific survival (nMSS) and Fine and Gray cause-specific cumulative incidence of death (competing risk) is shown. Number of deaths is shown (events) per age group; Adolescents and Young Adult (AYA) versus older adults (Adult). Crude hazard ratio (HR), and adjusted HR are shown. HR were adjusted for: lactate dehydrogenase level, Eastern Cooperative Oncology Group performance status, distant metastasis in ≥3 organ sites, brain metastases and the presence of a BRAF V600 mutation.
* Sub-distribution HR, from the Fine and Gray model.

In AYAs 88.2% of all deaths was caused by melanoma, this was 75.1% in older adults, $p = 0.002$. DSS and a competing risk analysis were performed to further investigate the difference in OS between AYAs and older adults. The DSS was better in AYAs (HR 0.81; 95% CI 0.66-1.00 and adjHR 0.79; 95% CI 0.64-0.98). The competing risk analysis showed that the difference in OS between AYAs and adults could be explained by the occurrence of more non-melanoma-related deaths in the older patient group. When accounting for non-melanoma related death as a competing risk, there was no difference between AYAs and older adults; sub-distribution HR (sHR) 0.90 (95% CI 0.73-1.11), adjusted sHR 0.92 (95% CI 0.75-1.13).

When addressing the non-melanoma-related deaths, both the non-melanoma specific survival (nMSS) and the sHR for melanoma specific death in AYAs as compared to older adults were lower. This indicated that AYAs had a significantly lower HR and sHR of dying of a non-melanoma-related cause; HR 0.32 (95% CI 0.18-0.57) and adjHR 0.33 (95% CI 0.18-0.58), sHR 0.36 (95% CI 0.20-0.63) and adjusted sHR 0.37 (95% CI 0.21-0.67). In Table 3 both the crude and adjusted HR for survival of AYAs and older adults is shown.

Discussion

In the largest prospective cohort study thus far, we observed that on a nation-wide scale 5% of all advanced melanoma patients were AYAs. Furthermore, we showed that BRAF and NRAS mutations are age dependent, leading to more AYAs being treated with targeted therapy. As current treatment strategies for this age group are adapted from clinical trials that mostly include older adults, it is important to investigate advanced melanoma in this young patient group[3,4].

By studying baseline characteristics of AYAs and older adults, we found more female patients in the AYA group than in the older adult group. This could be explained by both biological gender differences[14,15] and behavior differences between male and female patients. Donley et al. recently suggested that an early age at menarche and a late age at menopause are associated with an increased risk of melanoma in postmenopausal women[16]. An earlier study by Smith et al. and a recent study by Støer et al., however, did not find convincing evidence that reproductive factors are associated with an increased risk of melanoma[17,18]. As a result that the women in these studies had a median age of 53.5 (Smith et al.) and 48 years (Støer et al.), future research might focus on reproductive factors and exogenous estrogen use in younger women to try to explain why advanced melanoma is more abundant in women than in men within AYAs, when compared to older adults.

We determined a significant difference in the distribution of histological subtype of melanoma between the two age groups of interest. AYAs more often presented with

SSM as compared to older adults, whereas older adults more frequently presented with NM. This is in accordance to what Verzì et al., Bartenstein et al. and Daryanani et al., previously described, although in cohorts with younger patients[9,19,20]. Moreover, we found that AYAs had significantly thinner tumors than older adults. Relatively more AYAs had a tumor with a Breslow depth below 2 mm, suggesting that these tumors were nevertheless more aggressive as they did develop into advanced disease. From earlier research we know that melanomas of patients under the age of 20 years were significantly thicker than the melanomas seen in their adult control group[21]. This difference could be explained by the relatively small number of patients under 20 years of age in our AYA group ($n = 6$).

Our analysis of mutational profiles revealed that the frequency of a BRAF V600 mutation declines with age, whereas the frequency of an NRAS mutation increases with age. Our findings support previous studies that found BRAF mutations to be more abundant in the AYAs than in older adults. A possible explanation is that melanomas without BRAF mutations require accumulation of high UV doses over time, further supported by the difference in anatomic site of primary tumor[13,22].

Moreover, AYAs were treated significantly more often with combination therapy (anti-CTLA-4 + anti-PD-1) than older adults. These differences might be explained by less fear of AEs in AYAs. However, we did not find differences in occurrence of grade 3-4 AEs between AYAs and older adults following immune checkpoint inhibition or BRAF/MEK-inhibition.

Interestingly, there was a difference in toxicity pattern following anti-CTLA-4 monotherapy and in combination with anti-PD-1. Following combination treatment, more AYAs developed a hepatitis when compared to older adults (9 out of 21 versus 36 out of 169). None of the 19 AYAs developed a colitis following initial anti-CTLA-4 monotherapy, while 71 out of 408 older adults did. It has been suggested that the gut microbiota can influence the occurrence of treatment-related AEs and even treatment efficacy after checkpoint inhibitor therapy[23]. The Bacteroidetes phylum of the intestinal microbiota has been identified to be protective against anti-CTLA-4-induced colitis by stimulating the differentiation of regulatory T cells, thereby limiting inflammation[24]. The intestinal microbiota changes throughout the human lifetime. It is unclear, however, whether AYAs have a higher proportion of Bacteroidetes than older adults, as recent studies have shown contradicting results[25,26]. Based on our findings, we encourage researchers of the intestinal microbiota to incorporate age dependent differences in their results.

Increased age is associated with changes in host immunity that could impact the effectiveness of checkpoint inhibition. With advancing age, the immune system

remodels and declines, predisposing older adults and elderly to a higher risk of infections, autoimmune diseases and malignancies as compared to younger adults[27]. The naïve T cell compartment declines 2-5-fold between the ages of 30 and 70 years old, and the ability to establish immunological memory to newly introduced antigens is compromised[28,29]. Furthermore, it was shown that CD4+ T cells of patients ≤ 50 show more signs of activation, when compared to patients ≥ 65 years of age[30]. These factors can contribute to a less effective T cell immune response against melanoma cells after immunotherapy with checkpoint inhibition in older adults and elderly as compared to the younger population. However, we established that there was no difference in response, nor PFS between AYAs and older adults following immune checkpoint inhibition or targeted therapy. In addition, we showed that the favorable OS of AYAs as compared to older adults is due to an increased risk for adults to die from a non-melanoma-related cause.

Strengths of our dataset include the fact that all data are prospectively registered by trained data managers and is subsequently approved by the treating physician. Moreover, our analysis is based on nation-wide data. However, there are some limitations. During data collection treatment patterns for patients with advanced melanoma have changed. In the early stages of the DMTR dataset, tumor mutational status was not determined in all patients. Additionally, we might not have included all patients between 15 and 18 years of age, as some might not have been referred to the medical oncologist. Furthermore, we relied on data registered in the DMTR database, which was mostly of clinical origin. It would be interesting to combine the clinical results with data on immunological parameters in both the blood, tumor and stroma. Additionally, we did not have supplementary information on specific mutation signatures or whole genome sequencing data to compare AYAs with older adults. In accordance with our current data, both Krauthammer and Wilmott report a high frequency of BRAF mutations in younger patients, while adults more frequently harbor NRAS mutations. Furthermore, Krauthammer et al. showed that the presence of NF1 and RASA2 mutations was also age dependent. The presence of NF1 mutations was associated with a UV-derived mutation signature, higher mutational load and lower disease-specific survival[31]. The article by Willmott et al. published whole genome sequencing data from 25 Australian AYA and 121 adult patients[13]. They found high mutational signatures of ultraviolet radiation damage in all patients. It would be interesting to compare whole genome sequencing data from AYAs and older adults from the Netherlands, or another non-high ambient ultraviolet radiation country, with the data from Australia. This could shed more light on the reasons why AYAs develop advanced melanoma at a younger age, and which (distinct) signaling pathways are used.

Conclusions

Concluding, we show, for the first time, on a nation-wide scale and with real-life data that the frequency of a BRAF V600 mutation declines with age, whereas the frequency of an NRAS mutation increases with age in patients with advanced melanoma. Furthermore, AYAs with advanced stage melanoma are more commonly afflicted with the histologic subtype of superficial spreading melanoma and have thinner tumors than older adults. Moreover, first-line treatment for AYAs is more often BRAF/MEK-inhibition or the combination of anti-CTLA-4 + anti-PD-1. These treatments lead to similar responses in both AYAs and older adults. Interestingly, toxicity patterns in AYAs were distinct with regard to anti-CTLA-4 monotherapy and combination treatment with anti-PD-1. Our study is an important step towards a better understanding of advanced melanoma in AYAs and might open doors for new studies with AYAs in order to improve daily clinical practice for advanced melanoma in the younger population.

Supplementary Materials

The following are available online at http://www.mdpi.com/2072-6694/12/8/2072/s1, Table S1. Grade 3-4 toxicity following initial systemic treatment. Subtypes of adverse events per treatment type are shown for Adolescents and Young Adults (AYA) and older adults (Adult). Figure S1: Types of initial systemic treatment initiated since the diagnosis of advanced melanoma. Cumulative number of targeted therapy and immune checkpoint inhibition initiated over time since July 2013 for Adolescents and Young Adults (AYA, solid line) and older adults (Adult, dotted line) is shown.

Author Contributions

Conceptualization, M.K.v.d.K., M.J.A.L.W., E.B. and E.K.; data curation, M.K.v.d.K.; formal analysis, M.K.v.d.K.; investigation, M.K.v.d.K. and M.J.A.L.W.; methodology, M.K.v.d.K., M.J.A.L.W. and E.B.; Supervision, E.B. and E.K.; Visualization, M.K.v.d.K.; writing—original draft, M.K.v.d.K. and M.J.A.L.W.; writing—review and editing, M.K.v.d.K., M.J.A.L.W., M.J.B.A., F.W.P.J.v.d.B., C.U.B., M.J.B.-S., M.P.D., J.W.B.d.G., G.A.P.H., D.P., R.S.v.R., K.P.M.S., A.J.t.T., A.A.M.v.d.V., G.V., M.W.J.M.W., J.B.A.G.H., A.J.M.v.d.E., E.B. and E.K.
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Conflicts of Interest

C.U. Blank reports receiving commercial research grants from Novartis, Bristol-Myers Squibb, and NanoString; is a paid advisory board member for Bristol-Myers Squibb, MSD, Roche, Novartis, GlaxoSmithKline, AstraZeneca, Pfizer, Lilly, GenMab, and Pierre Fabre; and holds ownership interest in Uniti Cars, Neon Therapeutics, and Forty Seven. M.J. Boers-Sonderen has served as an advisory board member for Bristol-Myers Squibb, Novartis, Merck and Pierre Fabre. A.J.M. van den Eertwegh has served as a speaker for Bristol-Myers Squibb and Novartis and an advisory board member for Bristol-Myers Squibb, MSD oncology, Amgen, Roche, Novartis, Sanofi, Pfizer, Ipsen, Merck, Pierre Fabre and has received research grants not related to this paper from Sanofi, Roche, Bristol-Myers Squibb, TEVA and Idera. J.W. de Groot is a paid consultant for Bristol-Myers Squibb and MSD. G.A.P. Hospers is an unpaid consultant/advisory board member for Bristol-Myers Squibb, MSD, Roche, and Novartis. D. Piersma has served as an advisory board member for Amgen, BMS and Pierre Fabre. A.A.M. van der Veldt is a paid consultant for Bristol-Myers Squibb, MSD, Novartis, Roche, Pfizer, Eisai, Ipsen, Pierre Fabre, Sanofi, and Bayer. J.B.A.G. Haanen is a paid consultant for AIMM, Neon Therapeutics, Immunocore, Vaximm, and Neogene Therapeutics, and reports receiving commercial research grants from Bristol-Myers Squibb, MSD, Novartis, and Neon Therapeutics. K.P.M. Suijkerbuijk has served as a consultant and/or advisory board member for Bristol-Myers Squibb, Novartis, MSD, Pierre Fabre and AbbVie and received honoraria/research support not related to this manuscript from Novartis, Roche and MSD. All paid to institution. H.W. Kapiteijn has served as a consultant and/or advisory board member for Bristol-Myers Squibb, Novartis, Merck, Pierre Fabre and has received research grants not related to this paper from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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CHAPTER
7

Comparing men and women with advanced melanoma

CHAPTER
7.1

Sex-Based Differences in Treatment with Immune Checkpoint Inhibition and Targeted Therapy for Advanced Melanoma: A Nationwide Cohort Study

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Monique K. van der Kooij, Olaf M. Dekkers, Maureen J.B. Aarts, Franchette W.P.J. van den Berkmortel, Marye J. Boers-Sonderen, Jan Willem B. de Groot, Geke A.P. Hospers, Djura Piersma, Rozemarijn S. van Rijn, Karijn P.M. Suijkerbuijk, Hans M. Westgeest, Astrid A.M. van der Veldt, Gerard Vreugdenhil, Sofie Wilgenhof, Michel W.J.M. Wouters, John B.A.G. Haanen , Alfonsus J.M. van den Eertwegh, Ellen Kapiteijn

Summary

Melanoma is a malignant form of skin cancer. The overall survival of patients with advanced stages of disease were initially low. Fortunately, in recent years systemic treatment with immunotherapy has prolonged survival. We set out to answer the question whether men and women with advanced melanoma differ in prognostic factors, tumor-response to immunotherapy, and treatment-related adverse events. All patients in the Netherlands were registered between July 2013 and July 2018. We showed that although clinical and tumor characteristics differ, the safety profile of immunotherapy is comparable. Furthermore, overall, a 10% survival advantage for women was seen. Following immunotherapy there was no survival difference.

Abstract

Recent meta-analyses show conflicting data on sex-dependent benefit following systemic treatment for advanced melanoma patients. We examined the nationwide Dutch Melanoma Treatment Registry (July 2013-July 2018), assessing sex-dependent differences in advanced melanoma patients (stage IIIC/IV) with respect to clinical characteristics, mutational profiles, treatments initiated, grade 3-4 adverse events (AEs), treatment responses, and mortality. We included 3985 patients, 2363 men (59%) and showed that although men and women with advanced melanoma differ in clinical and tumor characteristics, the safety profile of immune checkpoint inhibition (ICI) is comparable. The data suggest a 10% survival advantage for women, mainly seen in patients ≥60 years of age and patients with BRAF V600 mutant melanoma. Following ICI there was no survival difference.

Introduction

Immunotherapy is currently changing the landscape of oncology. Systemic treatment with immune checkpoint inhibition (ICI) targeting programmed cell death 1 (anti-PD-1) and cytotoxic T-lymphocyte antigen-4 (anti-CTLA-4) can overcome tumor-induced immunosuppression in advanced malignancies[1]. BRAF, NRAS and c-KIT mutations in melanoma have shown to be distinct clinic-pathological entities[2]. Targeted therapy with BRAF-inhibition has demonstrated clear antitumor activity in patients whose tumors harbor the characteristic BRAF V600E or V600K mutation[3,4]. The addition of a MEK-inhibitor has shown to lead to more (durable) clinical responses[5]. Interestingly, membrane-bound estrogen receptors were shown to be responsible for an increased activity of the RAS/BRAF/MEK axis[6].

Components of both the innate and the adaptive immune system are differently regulated in men and women. Female patients have a faster clearance of pathogens and greater vaccine efficacy, but are more prone to inflammatory and autoimmune diseases. Contrarily, men have an almost twofold greater risk of mortality from malignant cancers[7]. In oncologic patients, it was recently shown that women are prone to stronger immunoediting in early tumor development. ICI in a later stage could therefore have a reduced effect in women, as this treatment will reactivate T cells for immunologically invisible (neo)antigens[8]. Furthermore, several studies reported differences between men and women in (possible) biomarkers for the response to ICI, including; tumor mutational burden, neoantigen load, PD-L1 expression, DNA mismatch repair deficiency, cytotoxic T cell infiltration, gene-expression and mutational signatures, antigen presentation defects, sex hormones, and interferon signaling[9-20].

In recent years, studies investigating the sex-dependent magnitude of benefit following treatment with ICI showed contradicting results. The first study showed that men derived greater value from ICI as compared to women[21]. Two more recent meta-analyses included several comprehensive and updated studies. These analyses concluded that there was no clear association between sex and the efficacy of ICI in the treatment of advanced cancers, including melanoma[22,23]. A fourth meta-analysis focused on anti-PD-1/anti-PD-L1 treatment in patients with advanced and metastatic cancer, including melanoma. They also could not show an overall survival (OS) difference between male and female patients[24].

The previously mentioned meta-analyses included large randomized controlled trials, however, a vast proportion of patients with advanced melanoma treated in daily practice do not meet the in- and exclusion criteria of these trials[25,26]. Another limitation of these analyses was that the authors lacked additional information on

patient-specific data, including the distribution of known risk factors among men and women[27]; this is important as the comparison between men and women in the setting of a randomized controlled trial can still be confounded, as it is not sex that is randomized. Potential differences in these prognostic markers, and tumor response following treatment between male and female patients could indicate that sex should be taken into account in the assessment of risk versus benefit when making decisions about treatment strategies. Therefore, using our population-based cohort of unresectable stage IIIC and IV melanoma patients, we set out to answer the question whether men and women differ in baseline and tumor characteristics, first-line systemic treatments initiated and the safety and efficacy of targeted therapy and ICI.

Materials and Methods

Dutch Melanoma Treatment Registry

Since 2013, all advanced melanoma patients in the Netherlands are referred to one of 14 expert hospitals and data are prospectively registered in the DMTR (Dutch Melanoma Treatment Registry). To assure safety and quality of melanoma care in the Netherlands centralization of advanced melanoma patients and subsequent their registration in the DMTR was initiated[28]. Information on patients' baseline and tumor characteristics, treatment regimens, grade 3-4 treatment related AEs (according to the Common Terminology Criteria for Adverse Events, version 4.0), clinical outcomes and date of death are registered. These data are collected from patient files by trained data managers and approved by the treating physicians. The DMTR was approved by a medical ethical committee (METC Leiden University Medical Center, Leiden, The Netherlands, 2013) and is not considered subject to the Medical Research Involving Human Subjects Act.

Patients, Treatments and Outcome Definitions

Data on all patients diagnosed with unresectable stage III or IV melanoma in the Netherlands between July 2013 and July 2018 were retrieved, follow-up data cut-off was set at 1 March 2019. The patient with missing data on gender ($N = 1$) was excluded from the analysis. After describing the location of primary tumor in male and female patients, patients with mucosal and uveal melanoma were excluded ($N = 375$). Patients with a melanoma of unknown primary were included in the analyses.

First-line anti-cancer systemic treatment strategies were compared between men and women, and included: chemotherapy with dacarbazine, ICI with anti-CTLA-4 (ipilimumab), anti-PD-1 (nivolumab, or pembrolizumab), or combination treatment with anti-CTLA-4 and anti-PD-1 (nivolumab and ipilimumab), targeted therapy

with BRAF-inhibitors (vemurafenib, dabrafenib, encorafenib) and MEK-inhibitors (trametinib, cobimetinib, binimetinib), or "other". Safety analysis was based on comparison of grade 3-4 AEs, and death due to adverse events (grade 5). Clinical outcomes were collected for all patients. The best overall response (BOR) is the best evaluation that a patient received after initiation of treatment, until the start of new melanoma therapy, or the last follow-up visit; progressive disease (PD), stable disease (SD), partial response (PR), or complete response (CR). The overall response rate (ORR) is defined as the proportion of patients who have a PR or CR following therapy. Survival time for all patients was calculated from the date of diagnosis of advanced melanoma to the date of the last follow-up visit (censored observation) or date of death as a result of any cause.

Statistical Analysis

Continuous variables were compared using a t-test, and chi-squared tests for categorical variables. All statistical tests were two-sided, and a p value of less than 0.05 was considered statistically significant. Potential differences between treatment choices in men and women after correcting for the presence of a BRAF V600 mutation were analyzed.

Progression free survival (PFS), overall survival (OS) and disease specific survival (DSS) were used as measure of survival probabilities. The cumulative incidence competing risk method was used to estimate melanoma-related mortality risk. To estimate subdistribution Hazard Ratio (sHR) and corresponding 95% CIs, Fine and Gray competing risk models were used with melanoma-related death as event and non-melanoma related death as competing risk. Risk factors that were included in the Cox proportional hazard and competing risk models were: age, ECOG performance status (0, 1, or ≥2), LDH level (not elevated, elevated within 2× upper limit of normal, or strongly elevated >2× upper limit of normal), presence of brain metastases, presence of distant metastasis in ≥3 organ sites, and BRAF mutation (presence of targetable—BRAFV600E or BRAFV600K—mutation). Patients that received BRAF inhibition were assumed to have a targetable BRAFV600 mutation in their tumor. Additionally, patients were stratified in age-groups corresponding with presumed hormonal status; pre-menopausal (≤45), menopausal (46-59) and post-menopausal (≥60 years of age). The peri-menopausal status was defined around the mean age of menopause, which is 50-51 years in Western countries and is in accordance with previously published research[29-31]. The proportional hazards assumption was checked by visual inspection.

Crude HRs and adjusted HRs for the above-mentioned risk factors and treatment groups were estimated. To test whether sex HRs differed across subgroups, an interaction term between sex and the subgroup variable was used.

SPSS version 25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY, USA, IBM Corp) was used to perform the descriptive statistics, Cox regression, Pearson Chi-Square analysis and survival analysis according to the Kaplan-Meier's method to calculate risk estimates. STATA version 14.1 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, USA, StataCorp LP.) was used to calculate cumulative incidence function in the presence of the competing risk (non-melanoma related death). Figures were created in GraphPad Prism version 8.1.1 (GraphPad Software, La Jolla, CA, USA).

Results

Baseline Characteristics

4361 advanced melanoma patients were registered; after excluding patients with mucosal and uveal melanoma, 3985 patients were selected; 2363 men (59.3%) and 1622 (40.7%) women, see Figure 1.

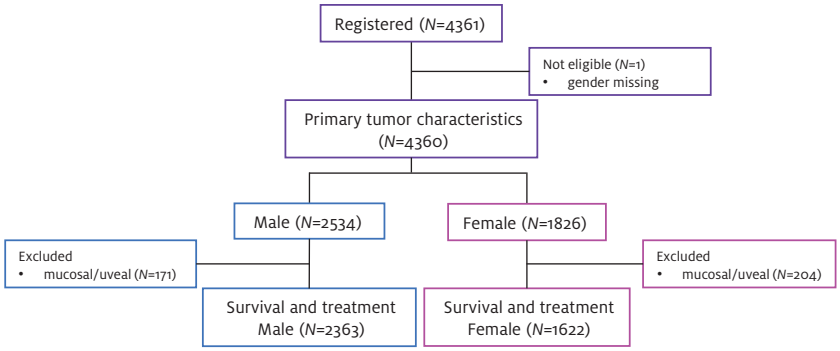

FIGURE 1 Patient selection for statistical analysis.

Clinical characteristics at time of advanced disease are shown in Table 1. Women were younger, with a median age of 63 versus 65 years ($p < 0.001$), had a lower M-stage (AJCC v7) at time of diagnosis ($p = 0.001$), and less often showed metastases in ≥ 3 organ sites (29.9 versus 34.8%, $p = 0.001$).

TABLE 1 Clinical and tumor characteristics of advanced cutaneous melanoma patients.

Characteristics at Baseline	Men N = 2363 (%)		Women N = 1622 (%)		*p* Value
Time since primary (months)	43	(0–841)	58	(0–603)	<0.001
Median age, year (range)	65	(15–97)	63	(17–96)	<0.001
Age categories					<0.001
≤45 years	218	(9.2%)	215	(13.3%)	
46–59 years	614	(26.0%)	451	(27.8%)	
≥60 years	1531	(64.8%)	956	(58.9%)	
ECOG PS					0.49
0	1086	(46.0%)	722	(44.5%)	
1	676	(28.6%)	460	(28.4%)	
≥2	313	(13.3%)	217	(13.4%)	
Unknown	287	(12.2%)	223	(13.7%)	
LDH					0.42
Normal (<250 U/l)	1365	(57.8%)	930	(57.3%)	
250–500 U/l	509	(21.5%)	348	(21.5%)	
>500 U/l	306	(12.9%)	196	(12.1%)	
Unknown	183	(7.7%)	148	(9.1%)	
M-stage					0.001
M1a	248	(10.5%)	218	(13.4%)	
M1b	263	(11.1%)	155	(9.6%)	
M1c	1804	(76.3%)	1194	(73.6%)	
Unknown	48	(2.0%)	55	(3.4%)	
Metastasis in ≥ 3 organ sites	822	(34.8%)	485	(29.9%)	0.001
Brain metastasis					
Yes	684	(28.9%)	428	(26.4%)	0.08
Symptomatic	487	(71.2%)	270	(63.1%)	0.005
Asymptomatic	197	(28.8%)	158	(36.9%)	
BRAF mutation					
V600 *	1117	(47.3%)	861	(53.1%)	0.001
V600E	866	(36.6%)	748	(46.1%)	
V600K	191	(8.1%)	71	(4.4%)	

ECOG PS: Eastern Cooperative Oncology Group Performance Status[32], LDH: Lactate dehydrogenase, M-stage: location of distant metastasis (M1a: skin and/or soft-tissue, M1b: lung, M1c: any other location), "*": mutation.

The anatomical location and clinical characteristics of the primary tumor are shown in Figure S1. In men the primary tumor was more often located in the head/neck and trunk (16 versus 9%), while in women it was more frequently located on the extremities (21 versus 36%). The primary melanomas of male patients were thicker, with more ulceration and were more frequently nodular. Female patients had a longer time gap between primary disease and development of advanced disease (58 versus 43 months).

Tumor Mutational Status

Overall, mutational pattern of the tumor differed between men and women, $p < 0.001$. Female patients more frequently harbored BRAF V600E mutant melanoma (46% versus 36%), while BRAF V600K and NRAS mutations were more prevalent in the tumors of male patients (8% versus 4% and 21% versus 18%, respectively). There was an age-dependent decrease in BRAF V600 mutations, while the percentage of patients harboring an NRAS mutation increased. In all age-groups BRAF V600E mutations were more frequently found in the tumors of female patients, whereas male patients more often carried a BRAF V600K or NRAS mutation, see Figure S2.

Initial Systemic Treatment Initiated

In 1736 men (74%) and 1180 women (73%) systemic therapy was the first-line treatment. Male patients more frequently received ICI (40% versus 35%), while targeted therapy was given more frequently to female patients (29% versus 26%). This difference was related to the presence of a BRAF mutation and disappeared after stratification; BRAF wild type ($p = 0.26$), BRAF V600 mutant ($p = 0.90$), and no BRAF mutational status determined ($p = 0.54$), see Figure 2.

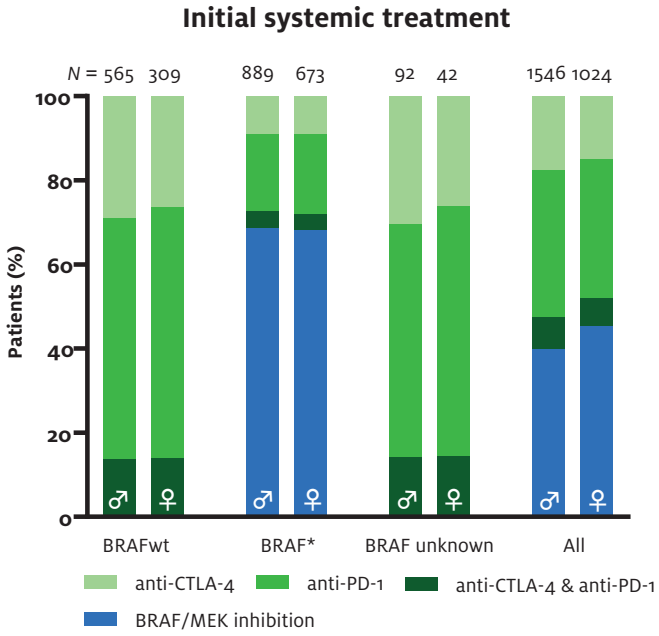

FIGURE 2 Initial systemic treatment with immune checkpoint inhibition and targeted therapy in male and female patients. "*": mutation.

Treatment Safety

Targeted Therapy (BRAF/MEK Inhibition)

Treatment with targeted therapy gave more grade 3-4 AEs in women, 25% versus 20%, respectively (p = 0.06) (Table 2). No clear difference in the type of AEs (Table 2) was found.

Immune Checkpoint Inhibition (Anti-CTLA-4, Anti-PD-1 and the Combination)

ICI with anti-CTLA-4 and anti-PD-1 resulted in similar percentages of AEs in men and women, which remained after adjusting for age. Furthermore, there was no difference in the type of AEs between these groups (Table 2). Adjustment for age made no material difference.

TABLE 2 Adverse events following systemic therapy.

Adverse Events	Men *N* (%)	Women *N* (%)	*p* Value
BRAF/MEK inhibition	614	463	
Grade 3–4	124 (20.2)	115 (25.1)	0.06
Skin/eye	56 (45.2)	55 (47.8)	
GI/Liver	41 (33.1)	38 (33.0)	
Other	54 (43.5)	39 (33.9)	
Grade 5	0	1 (0.2)	
Anti-CTLA-4	273	154	
Grade 3–4	87 (31.9)	49 (31.8)	0.99
GI/Liver	52 (59.8)	29 (59.2)	
Endocrine	20 (23.0)	12 (24.5)	
Skin	10 (11.5)	2 (4.2)	
Myelotoxicity	4 (4.6)	0	
Neurological/Uveitis	1 (1.1)	1 (2.0)	
Other	16 (18.4)	7 (14.6)	
Grade 5	2 (0.7)	0	
Anti-PD-1	513	324	
Grade 3–4	75 (14.6)	42 (13.0)	0.50
GI/Liver	24 (32.0)	16 (38.1)	
Endocrine	8 (10.7)	2 (4.8)	
Skin	5 (6.7)	6 (14.3)	
Renal	7 (9.3)	3 (7.1)	
Respiratory	9 (12.0)	4 (9.5)	
Myelotoxicity	2 (2.7)	0	
Neurological/Uveitis	2 (2.7)	0	
Other	30 (40.0)	19 (45.2)	
Grade 5	1 (1.4)	2 (4.9)	
Anti-CTLA-4 + anti-PD-1	120	70	
Grade 3–4	68 (56.7)	40 (57.1)	0.95
GI/Liver	48 (70.6)	28 (70.0)	
Endocrine	11 (16.2)	9 (22.5)	
Skin	7 (10.3)	4 (10.0)	
Renal	3 (4.5)	1 (2.5)	
Respiratory	4 (6.1)	4 (10.0)	
Myelotoxicity	1 (1.5)	0	
Neurological	2 (2.9)	2 (5.0)	
Other	13 (19.1)	10 (25.0)	
Grade 5	1 (0.8)	0	

Treatment Efficacy

Response rates (ORR; PR or CR) following ICI with either anti-CTLA-4 (20 versus 18%, $p = 0.62$) or anti-PD-1 (53 versus 51%, $p = 0.59$) were similar for men and women. However, men had lower ORRs compared to women following targeted therapy (52 versus 58%, $p = 0.07$) and combination treatment with anti-CTLA-4 + anti-PD-1 (51 versus 67%, $p = 0.06$), see Table S1. This difference in response remained after adjusting for the previously described prognostic factors, see Table S1.

Survival

Median OS was 59 weeks in male patients and 71 weeks in female patients. After adjusting for prognostic factors, adjHRs for women when compared to men were 0.92 (95% CI 0.84-0.99) for OS, 0.89 (95% CI 0.81-0.98) for DSS (0.92 (95% CI 0.83-1.01) when accounting for the competing risks) (Table 3).

Following targeted therapy, female patients had a longer PFS (adjHR 0.85, 95% CI 0.73-0.99) and a better OS (adjHR of 0.89, 95% CI 0.77-1.03) compared to male patients. There was no difference in survival following ICI monotherapy with; anti-CTLA-4, adjHR 0.86 (95% CI 0.66-1.10) or anti-PD-1, adjHR 1.11 (95% CI 0.89-1.38). Although the number of patients treated with combination therapy anti-CTLA-4 + anti-PD-1 was limited ($n = 190$), the point estimate suggests a possible survival advantage for women when compared to men HR 0.66 (95% CI 0.38-1.13).

When stratifying all patients across menopausal age categories, differences in adjusted HRs for OS and DSS were mainly seen in patients ≥60 years of age (Table S2). Furthermore, survival advantage of female patients treated with targeted therapy was also mainly seen in the postmenopausal age group with adjusted HRs for PFS 0.72 (95% CI 0.58-0.89), OS 0.69 (95% CI 0.57-0.85) and DSS 0.75 (95% CI 0.59-0.94). In the younger age groups, there were not enough patients treated with ICI to reliably estimate adjHRs (Table S2).

TABLE 3 Survival of female compared to male patients following initial systemic treatments.

Treatment groups		Events/Total (N)			
		Men	Women	HR (95% CI)	adjHR (95% CI)
All patients					
	OS	1446/2363	949/1622	0.90 (0.83–0.98)	0.92 (0.84–0.99)
	DSS	1109/2363	709/1622	0.88 (0.80–0.96)	0.89 (0.81–0.98)
	Comp. risk	1109/2363	709/1622	0.90 (0.82–0.98)	0.92 (0.83–1.01)
Initial treatment					
BRAF/MEK inhibition					
	OS	457/614	328/463	0.90 (0.78–1.04)	0.89 (0.77–1.03)
	DSS	375/614	259/463	0.87 (0.74–1.02)	0.86 (0.73–1.01)
	Comp. risk	375/614	259/463	0.89 (0.76–1.05)	0.90 (0.76–1.06)
	PFS	416/614	292/463	0.86 (0.74–1.00)	0.85 (0.73–0.99)
Anti-CTLA-4					
	OS	187/273	102/154	0.89 (0.70–1.13)	0.86 (0.66–1.10)
	DSS	153/273	83/154	0.88 (0.67–1.15)	0.84 (0.64–1.11)
	Comp. risk	153/273	83/154	0.88 (0.68–1.15)	0.84 (0.63–1.12)
	PFS	247/273	140/154	0.96 (0.78–1.19)	0.95 (0.77–1.18)
Anti-PD-1					
	OS	210/536	138/336	1.07 (0.86–1.32)	1.11 (0.89–1.38)
	DSS	156/536	106/336	1.10 (0.86–1.41)	1.13 (0.88–1.46)
	Comp. risk	156/536	106/336	1.10 (0.86–1.41)	1.14 (0.87–1.49)
	PFS	333/536	211/336	1.07 (0.90–1.27)	1.07 (0.90–1.28)
Anti-CTLA-4 + anti-PD-1					
	OS	50/120	18/70	0.66 (0.38–1.13)	-
	DSS	47/120	15/70	0.58 (0.32–1.04)	-
	Comp. risk	47/120	15/70	0.58 (0.32–1.04)	-
	PFS	77/120	32/70	0.74 (0.48–1.12)	-

Events and total number of men and women is shown, followed by hazard ratio and corresponding 95% confidence interval, and the adjusted hazard (adjHR) ratio with 95% confidence interval for overall survival (OS), disease specific survival (DSS), and progression free survival (PFS). Hazard ratios were adjusted for: sex, age, ECOG performance status, LDH, ≥3 organ sites affected, the presence of brain metastases, and BRAF V600 mutation status. Only for patients treated with targeted therapy was the BRAF V600 mutational status not included in the Cox proportional hazard model. Due to the limited number of patients treated with combination therapy with anti-CTLA-4 and anti-PD-1, no adjHRs were calculated for this subgroup of patients.

BRAF V600 Mutation

OS advantage of women could only be observed in patients harboring a BRAF V600 mutation, adjHR 0.87 (95% CI 0.78-0.98) and remained after restriction to BRAF V600E mutations. The same pattern could be observed for DSS, see Figure 3.

Risk Factors for Overall Survival in Male and Female Patients

Forest plots of the subgroup analyses of the sex difference for OS are shown in Figure 4, including *p*-values for interaction of these subgroups with sex. The female patient survival advantage was observed in the majority of subgroups, including the subgroup of female patients that was not systemically treated. Women seemed to have equal advantage with high or low tumor-burden; the HR remained similar in patients with <3 versus ≥3 organs involved and showed only a slight decrease in patients with a higher LDH serum level.

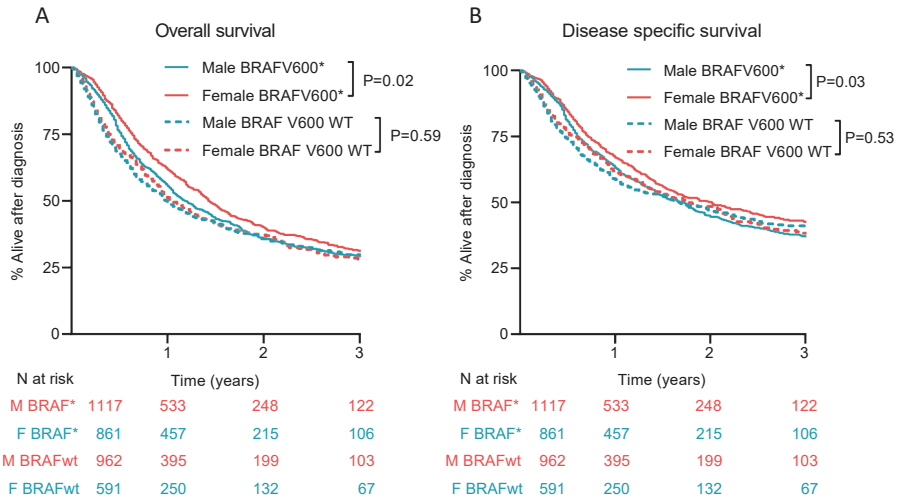

FIGURE 3 Overall and disease specific survival in men and women stratified by BRAF mutational status (**A**) Overall survival in years since diagnosis of advanced melanoma in patients with a BRAF V600 mutation (BRAFV600 */BRAF *) and patients proven to be BRAF V600 wild type (BRAF V600 WT/BRAFwt). (**B**) Disease specific survival in years is shown since diagnosis of advanced melanoma in patients with a BRAF V600 * and patients proven to be BRAF V600 WT. M = male, F = female, "*" = mutation.

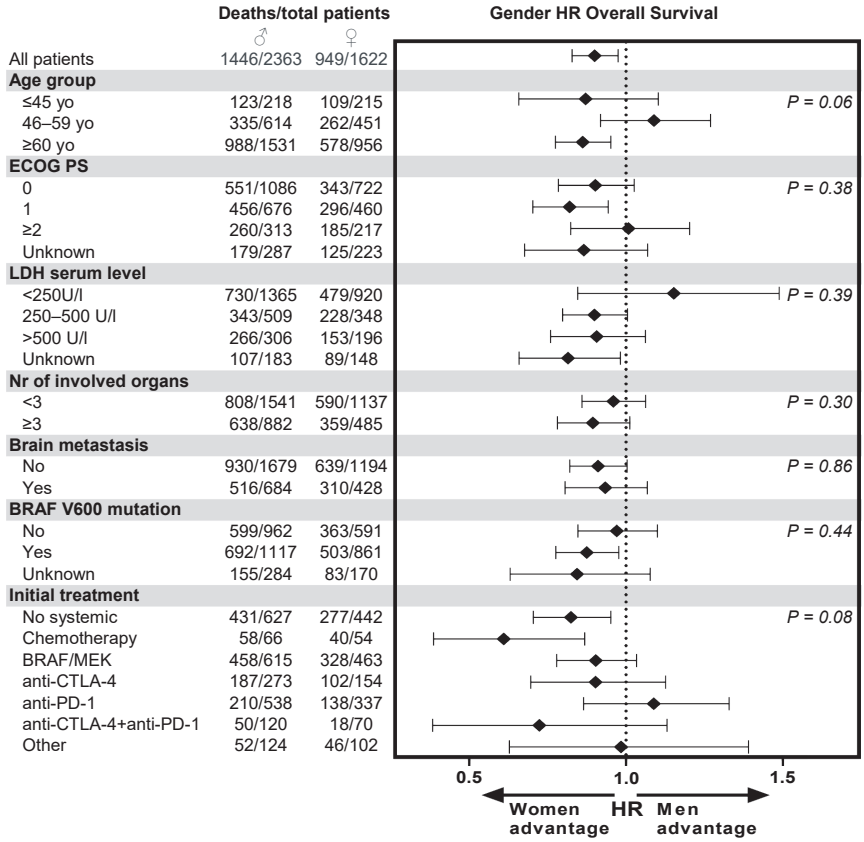

FIGURE 4 Subgroup analyses for overall survival. Subgroup analyses presented show crude sex HRs for overall survival. *p*-values presented show the statistical significance of the interaction term of the presented prognostic factor and sex in a Cox proportional hazard model.

Discussion

The data from this nation-wide study show that female patients with advanced melanoma have an OS advantage of approximately 10% over male patients. However, this difference appeared to be driven by the subgroups of postmenopausal women and female patients with a BRAF V600 mutant melanoma.

From previous research it is known that men, compared to women, are less likely to self-detect their melanomas[33] and make fewer visits to healthcare providers[34]. This could result in diagnostic delay in men, explaining the baseline differences found in our study. Corresponding with a diagnosis at an earlier time, female patients had thinner primary melanomas, less ulceration, and less nodular melanomas. Once

women developed advanced melanoma they had a lower M-stage with less organ sites affected by distant metastases. However, the time-gap between primary and advanced melanoma was longer in female patients. This indicates a less aggressive tumor proliferation in female patients or a stronger anti-tumor response in early tumor development[8].

Historically, the presence of a BRAF V600 mutation was associated with more aggressive tumor features and a shorter survival[35,36]. Due to the introduction of BRAF- and MEK-inhibition, this mutation has become a target for anti-tumor treatment. Our data show that advanced melanoma in women more frequently harbors a BRAF V600E mutation, while melanoma in men more frequently has a NRAS or BRAF V600K mutation. Our data strengthen data from previously published smaller cohorts[37-39].

The increased ratio of BRAF mutant melanomas in female versus male patients resulted in more targeted therapy initially being prescribed to female patients. Although this treatment did lead to more grade 3-4 AEs, it also yielded a higher ORR in women, which translated into a longer PFS.

The safety profiles of ICI were similar in men and women. Data on our 427 patients treated with anti-CTLA-4 contradicts previously published data on 140 patients by Valpione et al.[40], who reported that more AEs occurred in female patients.

Multiple retrospective and some prospective trials and meta-analyses have investigated sex as a prognostic factor for survival in (advanced) melanoma. Possible explanations for sex differences were: age at diagnosis, disease severity, tumor composition and infiltration, influence of estrogens in female patients, and overall longevity of women. Our current findings show that the survival advantage is mainly seen in the older (postmenopausal) age-group which supports the hypothesis that this might be due to female longevity. On the contrary, the observation that there was no difference in the efficacy of ICI over the different age-groups contradicts the influence of estrogens in female patients.

Before the introduction of ICI and targeted therapy, a pooled analysis of five EORTC randomized trials with metastatic melanoma showed that women had a better OS, DSS and PFS when compared to men. This difference decreased in female patients with more advanced disease[31]. These results were similar to a paper on the American SEER database, including melanoma patients with localized, regional, and metastatic disease[41]. Our study reports a female OS advantage in both patients with more and less advanced disease, in the era of ICI and targeted therapy.

A major strength of our population-based registry over the meta-analyses discussed in the introduction is that we also report data from patients with more advanced melanoma and a worse clinical performance score that do not meet the in- and exclusion criteria[25,26]. Another advantage of our registry is that we were able to adjust survival for patient baseline (tumor) characteristics and known risk factors. Furthermore, the data shown is from a more homogeneous group when compared to some meta-analyses that include patients irrespective of tumor type.

A limitation of our study is that data on hormonal status groups was based on age. Furthermore, not all patients progressed on their initial treatment before the start of a second line of systemic therapy. For example, treatment with targeted therapy could be given as an induction therapy. Therefore, data on ORR and PFS will be less reliable when compared to OS. The number of patients treated with combination treatment anti-CTLA-4 and anti-PD-1 was limited, therefore results on toxicity and efficacy of this treatment regimen have to be interpreted with caution. Additionally, as all patients in the Netherlands were included, systemic therapy could have been given as part of a clinical trial.

Conclusions

Our study shows that female advanced melanoma patients have an OS advantage of approximately 10% over male patients. Furthermore, women treated with targeted therapy have a better ORR and PFS, leading to a better OS in women with a BRAF V600 mutant melanoma over men. This difference was not seen in the patients without this mutation, nor in male and female patients initially treated with ICI.

The usage of a population-based registry with national coverage omits limitations from large phase III trials by also including patients that would not be eligible for studies. We encourage the use of this population-based data in the future to compare treatment choices, and to complement information that is provided by meta-analyses on drug safety and efficacy.

Supplementary Materials

The following are available online at https://www.mdpi.com/article/ 10.3390/ cancers13184639/s1, Figure S2: Mutational pattern of the tumor in men and women with advanced melanoma, stratified by age-groups, Table S1: Best overall response rate following systemic therapy, Table S2: Survival in different age categories.

Author Contributions

Conceptualization, M.K.v.d.K., O.M.D. and E.K.; Data curation, M.K.v.d.K.; Formal analysis, M.K.v.d.K.; Methodology, M.K.v.d.K., O.M.D. and E.K.; Resources, F.W.P.J.v.d.B., M.J.B.-S., J.W.B.d.G., G.A.P.H., D.P., R.S.v.R., K.P.M.S., H.M.W., A.A.M.v.d.V., G.V., S.W., M.W.J.M.W., J.B.A.G.H., A.J.M.v.d.E. and E.K.; Software, M.J.B.A.; Supervision, O.M.D. and E.K.; Visualization, M.K.v.d.K.; Writing-original draft, M.K.v.d.K.; Writing-review & editing, O.M.D., M.J.B.A., F.W.P.J.v.d.B., M.J.B.-S., J.W.B.d.G., G.A.P.H., D.P., R.S.v.R., K.P.M.S., H.M.W., A.A.M.v.d.V., G.V., S.W., M.W.J.M.W., J.B.A.G.H., A.J.M.v.d.E. and E.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The DMTR was approved by a medical ethical committee (METC Leiden University Medical Center, 2013) and is not considered subject to the Medical Research Involving Human Subjects Act.

Informed Consent Statement

Patient consent was waived due to the fact that the DMTR was not considered to be subject to the Medical Research Involving Human Subjects Act.

Conflicts of Interest

M.J.B.S. has served as an advisory board member for Bristol-Myers Squibb, Novartis, Merck and Pierre Fabre. A.J.M.v.d.E. has served as a speaker for Bristol-Myers Squibb and Novartis and an advisory board member for Bristol Myers Squibb, MSD oncology, Amgen, Roche, Novartis, Sanofi, Pfizer, Ipsen, Merck, Pierre Fabre and has received research grants not related to this paper from Sanofi, Roche, Bristol Myers Squibb, TEVA and Idera. J.W.B.d.G. is a paid consultant for Bristol Myers Squibb, MSD, Pierre Fabre, and Servier. G.A.P.H. is an unpaid consultant/advisory board member for Bristol

Myers Squibb, MSD, Roche, and Novartis. D.P. has served as an advisory board member for Amgen, Bristol Myers Squibb and Pierre Fabre. A.A.M.v.d.V. is a paid consultant for Bristol Myers Squibb, MSD, Novartis, Roche, Pfizer, Eisai, Ipsen, Pierre Fabre, Sanofi, and Bayer. J.H. is a paid consultant for AIMM, Neon Therapeutics, Immunocore, Vaximm, and Neogene Therapeutics, and reports receiving commercial research grants from Bristol Myers Squibb, MSD, Novartis, and Neon Therapeutics. K.P.M.S. has served as a consultant and/or advisory board member for Bristol Myers Squibb, Novartis, MSD, Pierre Fabre and AbbVie and received honoraria/research support not related to this manuscript from Novartis, Roche and MSD. All paid to institution. EK has served as a consultant and/or advisory board member for Bristol Myers Squibb, Novartis, Merck, Pierre Fabre and has received research grants not related to this paper from Bristol Myers Squibb. H.M.W. received travel expenses from Ipsen and honoraria from Astellas and Roche. No potential conflicts of interest were disclosed by the other authors. Role of the Funder: The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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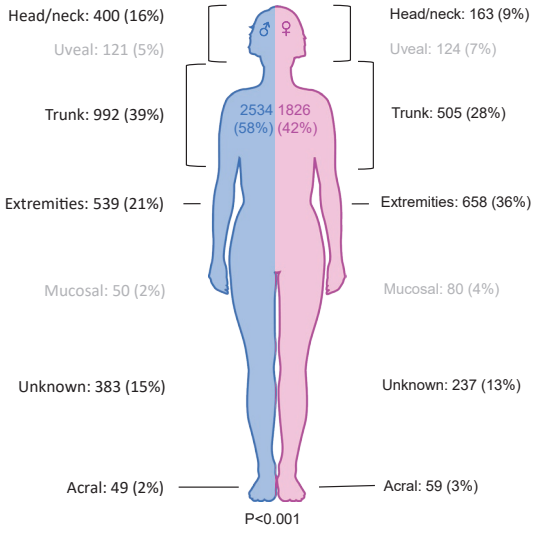

B

	Males	Females	*p*-value
Thickness			<0.001
≤1.00mm	236 (10.0%)	209 (12.9%)	
1.01-2.00mm	461 (19.5%)	398 (24.5%)	
2.01-4.00mm	614 (26.0%)	375 (23.1%)	
>4.00mm	518 (21.9%)	256 (15.8%)	
Unknown	534 (22.6%)	384 (23.7%)	
Ulceration			0.01
Present	711 (30.1%)	421 (26.0%)	
Absent	974 (41.2%)	690 (42.5%)	
Unknown	678 (28.7%)	511 (31.5%)	
Positive LN			0.15
Present	301 (12.7%)	175 (10.8%)	
Absent	1802 (76.3%)	1274 (78.5%)	
Unknown	260 (11.0%)	173 (10.7%)	
Distant metastasis			0.07
Present	429 (18.2%)	250 (15.4%)	
Absent	1852 (78.4%)	1318 (81.3%)	
Unknown	82 (3.5%)	54 (3.3%)	
Melanoma variant			<0.001
Superficial spreading	954 (40.4%)	682 (42.0%)	
Nodular	569 (24.1%)	289 (17.8%)	
Other	168 (7.1%)	171 (10.5%)	
Unknown	672 (28.4%)	480 (29.6%)	

FIGURE S1 Characteristics of primary tumors of male and female patients.

(A) Anatomical location of the primary tumor.

(B) Characteristics of the primary cutaneous melanomas, excluding uveal and mucosal melanoma

CHAPTER
7.2

Failure to validate existing clinical prediction scale for response to PD-1 monotherapy in advanced melanoma in national cohort study

Br J Cancer (2022)

Monique K. van der Kooij, Arjen Joosse, Karijn P.M. Suijkerbuijk, Maureen J.B. Aarts, Franchette W.P.J. van den Berkmortel, Christian U. Blank, Marye J. Boers-Sonderen, Alfonsus J.M. van den Eertwegh, Jan Willem B. de Groot, John B.A.G. Haanen, Geke A.P. Hospers, Djura Piersma, Rozemarijn S. van Rijn, Astrid A.M. van der Veldt, Gerard Vreugdenhil, Hans M. Westgeest, Michel W.J.M Wouters, Olaf M. Dekkers, Ellen Kapiteijn

Introduction

Treatment with targeted therapy and immune checkpoint inhibitors has significantly improved survival of patients with advanced melanoma. Unfortunately, a large proportion of patients are either primary non-responders or will eventually develop secondary resistance.

In 2017, Nosrati and colleagues published a prediction scale in the British Journal of Cancer, which included five clinical parameters that were associated with lower response to anti-PD-1 treatment; female sex (1 point), age <65 years (1 point), history of ipilimumab (anti-CTLA-4) treatment (2 points), elevated lactate dehydrogenase (LDH) (1 point), and the presence of liver metastasis (2 points)[1]. This study used a derivation cohort of 228 patients treated in California, and a validation cohort of 87 patients treated in Switzerland. The primary outcome measure was best tumor response to treatment evaluated using computed tomography at 12 and 16 weeks after the first administration of anti-PD-1 monotherapy, and every 12 weeks thereafter.

The aim of this correspondence is to validate the prediction scale, published by Nosrati and colleagues.

Patients and methods

Registry

Since 2013, all patients with advanced melanoma in the Netherlands are referred to one of 14 expert hospitals and data are prospectively registered in the Dutch Melanoma Treatment Registry (DMTR).

Data are collected from patient files by trained data managers and approved by the treating physicians. In compliance with Dutch regulations, the DMTR was approved by a medical ethical committee (METC Leiden University Medical Center, 2013) and is not considered subject to the Medical Research Involving Human Subjects Act.

Patients and data

We extracted data for all patients registered between July 2013 and July 2018. Patients without response evaluation scans ≥10 weeks after start of treatment (n=284), with missing data on the clinical parameters included in the prediction scale (n=134), or with uveal melanoma (n=17) were excluded. Baseline characteristics at the start of anti-PD-1 monotherapy were collected, including serum LDH, age, sex, previous treatments

and the presence of liver metastasis. Response was defined as complete response (CR) or partial response (PR), based on clinical judgement of the medical team.

Results

Between July 2013 and July 2018, 1292 patients started anti-PD-1 treatment and met inclusion criteria. Baseline characteristics are summarized in Table 1A, including differences between the derivation cohort of Nosrati et al. and our national cohort. Patients' sex was more equally distributed in our cohort. Furthermore, our cohort contained more patients with WHO performance score >0, fewer patients with elevated LDH levels, fewer BRAF wild type melanoma, and fewer patients who were previously treated with ipilimumab or targeted therapy.

Table 1B presents all clinical parameters that were found to be significantly associated with response to anti-PD-1 monotherapy in the univariate analysis by Nosrati et al. Both prior ipilimumab treatment (OR=0.73 95%CI; 0.56-0.96, *P*=0.02) and the presence of liver metastases (OR=0.70 (95% CI 0.54-0.90), *P*=0.006) were also found to be significantly correlated with lack of response to treatment in our cohort.

Figure 1 shows the predictive value of the clinical prediction scale of 0-7 points of Nosrati et al. With an AUC of 0.55 (*p*=0.001) this scale did not predict response to anti-PD-1 monotherapy in our cohort.

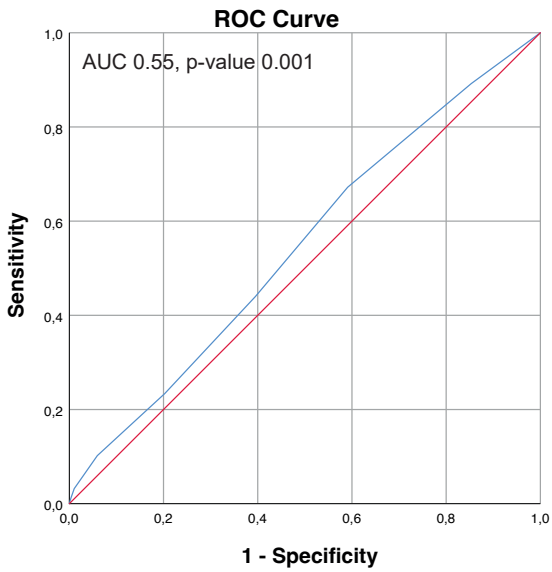

FIGURE 1 Receiver operation characteristics curve of the clinical prediction scale of 0-7 points of Nosrati et al. to predict response to anti-PD-1 monotherapy in our cohort.

TABLE 1 Baseline characteristics and performance of prediction scale. (**A**) Comparison of baseline characteristics between validation cohort of Nosrati and colleagues and our cohort, using descriptive statistics. (**B**) Significance of predictive clinical parameters of Nosrati's univariate analysis in our cohort, calculated using logistic regression.

Variable	Nosrati Number (%)	van der Kooij Number (%)
Age, years		
Mean +/- SD	62.5 +/- 13.1	63.3 +/- 12.9
Age <65 years	126 (55.3)	627 (48.5)
Sex		
Male	148 (64.9)	771 (59.7)
Female	80 (35.1)	521 (40.3)
Primary site		
Cutaneous	200 (87.7)	1032 (79.9)
Mucosal	13 (5.7)	43 (3.3)
Acral		32 (2.5)
Eye		
Unknown	15 (6.6)	185 (14.3)
ECOG		
0	157 (68.9)	725 (56.1)
1	65 (28.5)	419 (32.4)
2	5 (2.2)	58 (4.5)
3	1 (0.4)	6 (0.5)
Unknown		84 (6.5)
LDH		
Normal	150 (65.8)	939 (72.7)
Elevated	78 (34.2)	353 (27.3)
BRAF mutation		
Negative	162 (72.0)	619 (47.9)
Positive	63 (28.0)	626 (48.5)
Unknown	3 (1.3)	47 (3.6)
Liver metastasis		
No	160 (70.2)	968 (74.9)
Yes	68 (29.8)	324 (25.1)
Lung metastasis		
No	94 (42.1)	595 (46.1)
Yes	132 (57.9)	678 (52.5)
Unknown		19 (1.4)
Brain metastasis		
No	178 (78.1)	961 (74.4)
Yes	50 (21.9)	294 (22.8)
Unknown		37 (2.8)
Prior ipilimumab		
No	**81 (35.5)**	**1021 (79.0)**
Yes	147 (64.5)	271 (21.0)
Prior targeted therapy		
No	**174 (76.3)**	**1144 (88.5)**
Yes	54 (23.7)	148 (11.5)

	ORR (%)	OR (95% CI)	*P* value
Total cohort	49.8	NA	NA
Age ≥65 years	49.7	Ref.	Ref.
Age <65 years	50.3	1.03 (0.82-1.28)	0.82
Normal LDH	51.6	Ref.	Ref.
Elevated LDH	45.7	0.79 (0.62-1.01)	0.06
Male sex	50.5	Ref.	Ref.
Female sex	49.3	0.96 (0.77-1.19)	0.69
No prior ipilimumab	51.6	Ref.	Ref.
Prior ipilimumab	43.9	0.73 (0.56-0.96)	0.02
No liver metastasis	52.2	Ref.	Ref.
Liver metastasis	43.3	0.70 (0.54-0.90)	0.006

Discussion

We could not confirm the predictive value of the clinical prediction scale of 0-7 points for response to anti-PD-1 monotherapy as published by Nosrati et al. A possible explanation could be the significantly higher ORR in the derivation (63.3%) cohort from Nosrati et al. compared to our cohort (49.8%), which could have led to an initial overestimation of the predictive value of their prediction scale. Additionally, our cohort differed from the group treated by Nosrati et al. when comparing the pre-treatment. More patients received prior targeted therapy in our cohort, while more patients received prior ipilimumab treatment in the group from Nosrati et al. Therefore, our cohort more closely resembles the current clinical setting where ipilimumab is less frequently given as a first line monotherapy for patients with advanced melanoma.

Although the prediction scale could not be validated in our cohort, we did show that prior ipilimumab treatment and the presence of liver metastases was associated with a smaller response chance. This lack of response in the group of patients that has been pre-treated with ipilimumab could be due to the fact that patients who already progressed on prior immune checkpoint inhibition have a primary or acquired resistance to this type of treatment[2]. And therefore might also be less susceptible to a second line of immunotherapy.

In recent years, multiple meta-analyses have been published investigating the sex-dependent magnitude of benefit following treatment with immune checkpoint inhibition. The first study showed that men have more benefit from immune checkpoint inhibition, including anti-PD-1[3], whereas the latter three showed no difference in efficacy and overall survival[4-6]. Our study supports the findings that sex on itself is not a predictor for response to anti-PD-1 treatment.

Failure to validate the prediction scale by Nosrati et al. indicates that response to anti-PD-1 monotherapy cannot only be predicted by clinical parameters, but is influenced by other factors. Examples currently being studied include tumor-intrinsic factors, immune cells and cytokines both in tumor tissue and blood[7,8] and include more readily available blood parameters, such as LDH, S100B, absolute leukocyte, lymphocyte, neutrophil counts and their ratios[9-11]. While further research on predictive models is encouraged, validation of these models in sufficiently large independent cohorts is of even more importance to test robustness and clinical applicability.

Additional information

Authors' contributions

Conceptualization, M.K.v.d.K., O.M.D. and E.K.; Data curation, M.K.v.d.K.; Formal analysis, M.K.v.d.K.; Methodology, M.K.v.d.K., O.M.D. and E.K.; Resources, F.W.P.J.v.d.B., M.J.B.-S., J.W.B.d.G., G.A.P.H., D.P., R.S.v.R., K.P.M.S., H.M.W., A.A.M.v.d.V., G.V., C.U.B., M.W.J.M.W., J.B.A.G.H., A.J.M.v.d.E. and E.K.; Software, M.J.B.A.; Supervision, O.M.D. and E.K.; Visualization, M.K.v.d.K.; Writing—original draft, M.K.v.d.K.; Writing—review & editing, O.M.D., M.J.B.A., F.W.P.J.v.d.B., M.J.B.-S., J.W.B.d.G., G.A.P.H., D.P., R.S.v.R., K.P.M.S., H.M.W., A.A.M.v.d.V., G.V., C.U.B., M.W.J.M.W., J.B.A.G.H., A.J.M.v.d.E. and E.K. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

The DMTR was approved by a medical ethical committee (METC Leiden University Medical Center, 2013) and is not considered subject to the Medical Research Involving Human Subjects Act.

Patient consent was waived due to the fact that the DMTR was not considered to be subject to the Medical Research Involving Human Subjects Act.

Conflict of Interest

M.J.B.S. has served as an advisory board member for Bristol-Myers Squibb, Novartis, Merck and Pierre Fabre. A.J.M.v.d.E. has served as a speaker for Bristol-Myers Squibb and Novartis and an advisory board member for Bristol Myers Squibb, MSD oncology, Amgen, Roche, Novartis, Sanofi, Pfizer, Ipsen, Merck, Pierre Fabre and has received research grants not related to this paper from Sanofi, Roche, Bristol Myers Squibb, TEVA and Idera. C.U.B. has served as ab advisory board member for Bristol-Myers Squibb, MSD, Roche, Novartis, GlaxoSmithKline, AstraZeneca, Pfizer, Lilly, GenMab, and Pierre Fabre, and reports to have ownership interests in Uniti Cars, Neon Therapeutics, and Forty Seven, and received commercial grants from Novartis, Bristol-Myers Squibb, and NanoString. J.W.B.d.G. is a paid consultant for Bristol Myers Squibb, MSD, Pierre Fabre, and Servier. G.A.P.H. is an unpaid consultant/advisory board member for Bristol Myers Squibb, MSD, Roche, and Novartis. D.P. has served as an advisory board member for Amgen, Bristol Myers Squibb and Pierre Fabre. A.A.M.v.d.V. is a paid consultant for Bristol Myers Squibb, MSD, Novartis, Roche, Pfizer, Eisai, Ipsen, Pierre Fabre, Sanofi, and Bayer. J.H. is a paid consultant for AIMM, Neon Therapeutics, Immunocore, Vaximm, and Neogene Therapeutics, and reports receiving commercial research grants from Bristol Myers Squibb, MSD, Novartis, and Neon Therapeutics. K.P.M.S.

has served as a consultant and/or advisory board member for Bristol Myers Squibb, Novartis, MSD, Pierre Fabre and AbbVie and received honoraria/research support not related to this manuscript from Novartis, Roche and MSD. All paid to institution. EK has served as a consultant and/or advisory board member for Bristol Myers Squibb, Novartis, Merck, Pierre Fabre and has received research grants not related to this paper from Bristol Myers Squibb. H.M.W. received travel expenses from Ipsen and honoraria from Astellas and Roche. No potential conflicts of interest were disclosed by the other authors. Role of the Funder: The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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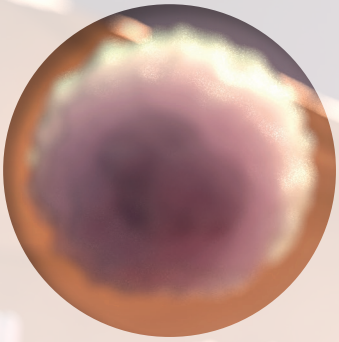

PART

III

Adoptive cell therapy in advanced melanoma

CHAPTER
8

Low-dose Interferon-Alpha Preconditioning and Adoptive Cell Therapy in Patients With Metastatic Melanoma Refractory to Standard (Immune) Therapies: A Phase I/II Study

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Els M.E. Verdegaal*, Monique K. van der Kooij*, Marten Visser, Caroline E. van der Minne, Linda de Bruin, Pauline M. Meij, Anton Terwisscha van Scheltinga, Marij J.P. Welters, Saskia J.A.M. Santegoets, Noel F.C.C. de Miranda, Inge C.F.M. Roozen, Gerrit-Jan Liefers, Ellen Kapiteijn**, Sjoerd H. van der Burg**

* joint first authors, ** joint senior authors.

Abstract

Background: Adoptive cell therapy (ACT) with tumor-reactive T cells has shown consistent clinical efficacy. We evaluated the response to ACT in combination with interferon alpha (IFNa) preconditioning in patients with stage IV metastatic melanoma, most of which were progressive on cytotoxic T-lymphocyte-associated protein 4 and/or programmed cell death protein 1 checkpoint blockade therapy.

Methods: Thirty-four patients were treated with *ex vivo* expanded tumor reactive T cells, derived from mixed lymphocyte autologous tumor cultures, or with autologous tumor-infiltrating lymphocytes and evaluated for clinical response. Clinical and immunological parameters associated with response were also evaluated.

Results: Best overall response defined as clinical benefit, comprising either complete response, partial response or stable disease >6 months, was observed in 29% of the patients. Forty-three percent of the 14 immunotherapy-naïve patients and 20% of the 20 patients progressive on prior immunotherapy benefited from ACT. The overall survival (OS) was 90% versus 28.6% at 1 year and 46.7% versus 0% at 3 years follow-up, of responder and non-responder patients, respectively. Median OS was 36 versus 7 months, respectively. IFNa pretreatment resulted in leukopenia, neutropenia and lymphopenia, which was sustained during the treatment in clinical responders and associated with response. Differences in antigen specificity, but not in phenotype, cytokine profile or CD8+ T cell number of the ACT products correlated with clinical response. Cross-reactivity of the ACT products to one or more allogeneic human leukocyte antigen-matched melanoma cell lines was associated with short OS after treatment while the ACT products of very long-term survivors showed no cross-reactivity but recognized patient-specific neoantigens.

Conclusion: This study demonstrates that ACT in combination with a mild IFNa preconditioning regimen can induce clinical benefit even in immunotherapy pretreated patients, although with lower success than in immunotherapy-naïve patients. ACT products comprising neoantigen reactivity may be more effective.

Introduction

The emergence of several new treatment options including targeted and checkpoint-blocking therapy for melanoma has dramatically improved the response rate from a very poor median survival time of 6-9 months to almost 2 years[1]. Nevertheless, almost half of the patients do not respond or eventually become refractory to these therapies[2-6]. Adoptive cell therapy (ACT) offers an additional treatment option for patients presenting with standard treatment refractory progressive disease (PD). ACT involves the reinfusion of *ex vivo* expanded autologous tumor-reactive T cells (TRT) or tumor infiltrating T cells (TIL) and is proven to be a very effective treatment modality for solid tumors resulting in an objective response rate of up to 50% in melanoma when administered after non-myeloablative conditioning by chemo-depletion and additional postinfusion of interleukin-2 (IL-2) in immunotherapy-naïve patients[7-10]. However, the response rate and overall survival (OS) considerably drop when patients are progressive on anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and/or programmed cell death protein 1 (PD-1) blockade prior to ACT treatment[11].

T cells used for infusion, that is, the ACT product, are generally obtained by *ex vivo* expansion of TIL, of which it is known that their abundance correlates with better survival in melanoma[12-14]. Alternatively, the ACT product can be formed by TRT, expanded from peripheral blood mononuclear cells (PBMC) by mixed lymphocyte tumor cell culture (MLTC)[15,16], requiring an established tumor cell line for repeated *ex vivo* stimulation, which is not feasible for most patients.

Previous ACT trials demonstrated the need for chemotherapy-driven lymphodepletion prior to T cell infusion and concomitant administration of high-dose IL-2 to obtain clinical success[17-19]. A considerable reduction of the toxicity associated with these protocols could be obtained by reduction of the postinfusion IL-2 dose[10,20-22]. In an interim analysis, we showed that clinical benefit can also be obtained when low-dose interferon alpha (IFNa) is used as a very mild and safe preconditioning and T cell supporting regimen[15].

Here, we report the data of the complete trial in which we investigated the safety and feasibility to treat patients with metastatic melanoma with adoptively transferred T cells in combination with IFNa. We dissected the effect of pretreatment clinical parameters, IFNa conditioning and the phenotypical as well as antigen-specificity characteristics of the ACT product in order to determine their association with clinical response.

Materials and Methods

Patient selection

Patients were eligible if 18 years or older with histologically proven stage IV or irresectable stage III cutaneous melanoma, with a WHO performance status 0-2 and a life expectancy of at least 6 months. Patients had PD at the start of treatment and systemic treatment had to be discontinued for 4 weeks in case of chemotherapy, radiotherapy or immunotherapy and 2 weeks in case of targeted therapy (BRAF/MEK inhibitors). At least one resectable or bioptable lesion was required for establishment of a tumor cell line and/or TIL culture and at least one additional measurable target lesion was required for response evaluation. Patients with asymptomatic or neurologically stable brain metastases were eligible for this study. Exclusion criteria were clinically significant heart disease (New York Heart Association class III or IV), active immunodeficiency or autoimmune disease, other malignancy within 3 years prior to entry into the study, a known allergy to penicillin or streptomycin or seropositivity for hepatitis B/C, HIV, HTLV or *Treponema pallidum*.

Study design

All patients were treated with autologous T cells in combination with IFNa. Low-dose IFNa injections (3 million units subcutaneous daily) were started 1 week (wk -1) before the first T cell infusion (wk 0) and continued for a total period of 12 weeks. T cell infusions were given intravenously with a 3-week interval. Patients were treated in three increasing dose cohorts of 1-2.5×10^8, 2.5-5×10^8 or 7.5-10×10^8 T cells per infusion for cohort I, II and III, respectively. Cryopreserved T cells were thawed and administered intravenously over a time period of 30-60 min.

Before and at several time points after infusions heparinized venous blood was collected and isolated PBMC as well as serum/plasma samples were cryopreserved until further analysis.

Before start of treatment and after three T cell infusions, the tumor response was evaluated by physical examination and imaging studies (CT and/or MRI) according to the Response Evaluation Criteria In Solid Tumors (RECIST) V.1.0 and V.1.1. Patients were admitted to the hospital for only 24 hours after the T cell infusions for observation. The primary objective was to evaluate the safety of the combination of T cells with low-dose IFNa, which was assessed using the NIH Common Terminology Criteria for Adverse Events (CTCAE) V.2.0 and V.4.0. Secondary objectives were clinical response evaluation and analysis of immunological parameters.

Generation of T cell products for infusion

Patients were treated with PBMC-derived TRT obtained by MLTC, as previously described[15]. These cultures required the use of an established autologous tumor cell line, which was not available for all patients. Alternatively, patients received TIL, which were readily available for each patient and cultured from a small resected tumor sample essentially using a previously described protocol[23] (Online Supplementary Figure 1). TIL were cultured in T cell medium (Iscoves Modified Dulbecco's Medium (IMDM) with penicillin (100 IU/mL), streptomycin (100 µg/mL) and L-glutamine (4 mM) (all from Life Technologies, Breda, The Netherlands), and 7.5% heat inactivated pooled human serum (Sanquin, Bloodbank, Amsterdam, The Netherlands) supplemented with IL-2 1000 IU/mL (Proleukin/Aldesleukin, Novartis, Arnhem, The Netherlands) for a total period of 14-21 days. Next, the TIL were expanded according to the described Rapid Expansion Protocol[23] for another 14 days before harvesting and cryopreservation, until further use. The production and batch release were performed under full Good Manufacturing Practices compliance (GMP).

Cell line generation and culture

Autologous melanoma cell lines were established in our GMP facility from resected tumor tissue as described previously[15]. All other melanoma cell lines were established in the laboratory of Medical Oncology (LUMC, Leiden, The Netherlands) except for melanoma cell lines FM3 and FM6 which were provided by P. Thor Straten, Copenhagen, Denmark. BLM was obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands), and MZ7.4-mel obtained from J. Gutenberg University (Mainz, Germany). Authentication of the cell lines was performed by human leukocyte antigen (HLA)-genotyping at the Department of Immunohematology and Bloodbank of the LUMC and they were regularly tested to be mycoplasma negative. All melanoma cell lines were cultured in tumor cell medium (ie, Dulbecco's minimal essential medium (Life Technologies) with 8% heat inactivated fetal calf serum (FCS), penicillin (100 IU/mL), streptomycin (100 µg/mL) and L-glutamine (4 mM) all from Life Technologies). Autologous EBV-LCL B cells and phytohemagglutinin (PHA)-stimulated T cell blasts (PHA-blasts) were established and cultured in B cell medium, that is, IMDM with 8% heat inactivated FCS, penicillin (100 IU/mL), streptomycin (100 µg/mL) and L-glutamine (4 mM). Epstein-Barr virus-transformed lymphoblastoid B cell lines (EBV-LCL) were used as APCs. These autologous EBV-LCL are known to process and present peptide both in HLA class I and II. The transformation was induced by incubation of patients' PBMC with supernatant of the marmoset B cell line containing infectious particles of EBV strain B95-8 for 1 hour at 37°C. Culture medium consisted of RPMI-1640, supplemented with 5 µg/mL PHA (Thermo Fisher Scientific), 10% FCS, L-glutamine (4 mM), penicillin (100 µg/mL) and streptomycin (100 µg/mL). Cells were

refreshed every 5-6 days with B cell medium and cultured for 3 weeks before being used as target cells.

Phenotypical analysis of PBMC

PBMCs collected before and after 1 week of IFNa treatment were thawed and divided into multiple samples that were stained with separate antibody panels for myeloid-derived suppressor cell (MDSC), inhibitory/memory, regulatory T cell and dendritic cell (DC) markers, respectively (Online Supplementary Table 1a). Dead cells were stained using Yellow ArC-Qdot585 (ThermoFisher, L34959).

Staining was carried out according to our standard protocols[24], washed with Fluorescence Activated Cell Sorting (FACS) buffer, fixed in 1% paraformaldehyde and analyzed using a LSRFortessa X20 (BD Biosciences). Staining of the regulatory T cell panel was conducted using the Transcription Buffer Set (BD Biosciences) as previously described[25]. FACS results were analyzed with BD FACSDiva software (V.8.02).

Cytokine analysis in serum/plasma

The serum/plasma concentration of homeostatic cytokines IL-7, IL-15 and IL-21 was analyzed using ELISA (R&D diagnostics; DY207, Biolegend; 435104, Mabtech; 3540-1 H-6), according to the manufacturer's instructions.

Phenotypical characterization of infused T cell

For detailed phenotypical characterization, reference vials of T cell batches used for infusion were thawed, counted and resuspended in FACS buffer consisting of phosphate-buffered saline+0.5% bovine serum albumin. Dead cells were stained using Yellow ArC-Qdot585 (ThermoFisher, L34959). Next, the T cells were divided into multiple samples and stained with separate antibody panels for inhibitory, homing, memory and regulatory T cell markers, respectively (Online Supplementary Table 1b). The staining was carried out according to our standard operating procedures as previously described[24], washed with FACS buffer, fixed in 1% paraformaldehyde and analyzed using a LSRFortessa X20 (BD Biosciences).

Functional characterization of the infused T cells

Tumor reactivity. The antigen specificity of the infusion product was tested against a broad panel of melanoma cell lines that were (partially) matched for at least one HLA class I allele with the corresponding patient. If available, autologous tumor cells were also tested. Briefly, 1.5×10^4 T cells (effector cells) were co-cultured with 3×10^4

target cells in a total volume of 150 µL B cell medium (ie, T cell medium with 8% FCS instead of human serum) in triplicate wells of a U-bottom 96-well plate. Medium alone and EBV-LCL B cells or PHA-blasts were used as negative controls and staphylococcal enterotoxin B (SEB, 0.5 µg/ mL) or PHA (5 µg/mL) were used as positive controls. After overnight incubation at 37°C, the supernatant was harvested to determine the interferon-gamma (IFNg) secretion as a measure of reactivity by ELISA (Sanquin) according to manufacturer's recommendations.

Neoantigen reactivity. To identify recognition of neoantigens derived from non-synonymous somatic mutations within expressed genes whole exome and RNA sequencing was performed and either 31-mer synthetic long peptides (SLPs) or 8-12-mer synthetic short peptides (SSPs) covering the mutation were manufactured as previously described[26]. Of note, in contrast to the SLP, the SSP were selected based on in silico prediction using the ISABELLA algorithm (ISA Pharmaceuticals, Leiden, The Netherlands). Next, T cells were incubated as described in the previous paragraph with target cells, that is, tumor cells or autologous B cells either unloaded or preloaded overnight with SLP pools or single peptides (10 µg/mL per peptide). Recognition of SSP was analyzed by direct addition of SSP (1 µg/mL per peptide) to the T cells. Medium alone or unloaded autologous B cells were included as negative controls and SEB (0.5 µg/mL) or PHA (5 µg/mL) as positive controls. Reactivity of T cells was measured after 24 hours co-incubation with target cells/peptides by IFNg secretion using ELISA (Sanquin).

Cytokine profile. To characterize the cytokine profile potentially released on activation of the infused T cells, T cells were stimulated with SEB (0.5 µg/mL) or PHA (5 µg/ mL) and if available with the autologous melanoma cell line as positive control and autologous EBV-LCL B cells, PHA-blasts or medium alone as negative controls. After incubation for 24 hours supernatant was harvested and used to analyze the cytokine production using the human Th1/Th2 cytometric bead array (BD Pharmingen). Specific cytokine production was defined by a cytokine concentration above the cut-off value (IFNg 50 pg/ mL; other cytokines 10 pg/mL) and >2× the concentration of the medium control[27].

Statistical analysis

Descriptive statistics were used to summarize patient baseline characteristics at start of treatment. Survival from start of treatment to progression and death was estimated according to the method by Kaplan-Meier using SPSS (V.25, IBM, released 2017).

Paired analyses between FACS data from PBMC samples of patients before and after 1 week of IFNa use were compared using Cytosplore V.2.1.5, R V.3.4.4, R studio V.1.1.442 and using the R-package cytofast[28]. Furthermore, paired and independent analyses were performed on the data generated by FACS analysis on both the T cell products

and the PBMCs by GraphPad Prism V.7.00 for Windows (GraphPad Software, La Jolla, California, USA) and SPSS. A D'Agostino and Pearson omnibus K2 test was performed to determine whether data were normally distributed within groups. To compare paired data following a normal distribution a paired t-test was used, when the assumption of normality was violated a Wilcoxon signed rank test was performed. For unpaired data following a normal distribution a unpaired t-test was used, when the assumption of normality was violated a Mann-Whitney U test was performed.

Results

Patient characteristics at baseline

Forty-one patients with progressive stage IV metastatic melanoma were included for treatment with ACT in combination with low-dose IFNa (ACT+IFNa) in our phase I/II trial between 2006 and 2018. All patients had PD before treatment and seven patients did not complete their full cycle of three infusions due to rapid disease progression. Thirty-four patients completed one full cycle of T cell infusions and were evaluated for safety/toxicity, clinical response and immunological parameters (Online Supplementary Figure 1). The patients were treated in three dose cohorts and received either TRT or TIL. The baseline characteristics of all patients are shown in Table 1. Details of start of treatment, (pre)-treatment regimens and response to treatment of individual patients are given in Online Supplementary Table 2. Comparison of previously described prognostic factors of worse OS did not differ at baseline between the different dose cohorts (Table 1) nor between patients treated with TRT versus TIL (Online Supplementary Table 3). Since several lines of systemic treatments are currently available for patients with stage IV metastatic melanoma, the majority of the evaluated patients (65%) received two or more lines of prior systemic therapies. Notably, the majority of TIL-treated patients (83%) was pretreated with checkpoint therapy (Online Supplementary Table 3). The percentage of patients with a confirmed brain metastasis was higher in the group of patients treated with TRT when compared with TIL-treated patients, 50% vs 29.2%, respectively (Online Supplementary Table 3). Univariate analyses of all baseline characteristics, including blood parameters previously reported to be important for immunotherapy, such as absolute leukocyte, lymphocyte, neutrophil counts and ratios thereof[29-33] showed that the WHO status as well as the leukocyte, monocyte and neutrophil counts as well as their ratios to lymphocytes were correlated with OS. In the multivariate analyses, only the WHO status, immunotherapy pretreatment and the monocyte-to-lymphocyte ratio (MLR) were associated with OS (Online Supplementary Figure 2a, Online Supplementary Table 4). Interestingly, except for MLR none of the other parameters was associated with time till progression after ACT in the multivariate analyses (Online Supplementary Figure 2b, Online Supplementary Table 5).

TABLE 1 Patient characteristics at baseline

		Cohort I (*n*=7)	Cohort II (*n*=21)	Cohort III (*n*=6)	*P* value*
Age mean (min-max)		50.43 (33-67)	54.24 (41-77)	46.50 (36-56)	0.24
Gender, *n* (%)	Male	7 (100)	14 (66.7)	5 (83.3)	
	Female	0 (0)	7 (33.3)	1 (16.7)	
LDH mean (min-max)		234.3 (144–477)	341.6 (136–973)	279 (166–446)	0.46
LDH level, *n* (%)	<250	5 (71.4)	11 (52.4)	2 (33.3)	
	250–500	2 (28.6)	7 (33.3)	4 (66.7)	
	>500	0 (0)	3 (14.3)	0 (0)	
WHO, *n* (%)	0	4 (57.1)	12 (57.1)	4 (66.7)	
	1	2 (28.6)	7 (33.3)	2 (33.3)	
	2	0 (0)	1 (4.8)	0 (0)	
	Missing	1 (14.3)	1 (4.8)	0 (0)	
Brain metastasis (confirmed), n (%)		5 (71.4)	6 (28.6)	1 (16.7)	0.07
Pretreatment, *n* (%)	BRAFi/MEKi	2 (28.6)	8 (38.1)	4 (66.7)	0.34
	Anti-CTLA-4 only	1 (14.3)	1 (4.8)	0 (0)	n.e.
	Anti-PD-1±anti-CTLA-4	(0)	12 (57.1)	6 (100)	**0.001**
Prior lines of systemic	0–2	7 (100)	14 (66.7)	1 (16.7)	**0.007**
therapies, *n* (%)	≥3	0 (0)	7 (33.3)	5 (83.3)	
TRT or TIL, *n* (%)	TRT	6 (85.7)	4 (19)	0 (0)	**0.001**
	TIL	1 (14.3)	17 (81)	6 (100)	
Responders†, *n* (%)		1 (14.3)	7 (33.3)	2 (33.3)	
		CR	CR, PR, 5×SD	2×SD	

*Statistically significant p values are indicated in bold, n.e.=not evaluable because n=2.

†Responders are defined by patients having CR, PR or SD.

CR, complete response; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; LDH, lactate dehydrogenase; PD-1, programmed cell death protein 1; PR, partial response; SD, stable disease; TIL, tumor infiltrating T cells; TRT, tumor-reactive T cells.

Clinical responses to ACT in combination with IFNa

T cell treatment was safe and well tolerated since no treatment-related events >3 grading according to CTCAE were observed (Online Supplementary Table 6). The adverse events were predominantly associated with the IFNa-treatment. A transient grade 3 leukopenia was observed in 4 out of 34 (11.8%) patients, grade 3 neutropenia in 5 of 34 (14.7%) patients and grade 3 lymphopenia in 7 of 34 (20.6%) patients, whereas most other patients experienced a mild leukopenia, neutropenia and lymphopenia.

Thirty-four of the patients with progressive stage IV metastatic melanoma could be evaluated for treatment response according to RECIST. Responder patients who obtained clinical benefit (CB) of treatment were defined as patients with complete response (CR), partial response (PR) or durable ≥6 months stable disease (SD) according to RECIST. From the 34 evaluable patients, 2 showed a CR, 1 PR and 7 displayed a prolonged SD. Thus, 10 out of 34 (29.4%) of the treated patients were defined as responder patients. The overall 3-year OS was 14.1% (95% CI 1.9 to 26.3) and 3-year overall progression-free survival was 8.8% (95% CI 0 to 18.4). Patients were treated in different dose cohorts but the responses were distributed among all doses. The two patients who obtained a CR both had elevated lactate dehydrogenase (LDH) (between 250 and 500 U/mL). One patient had a confirmed brain metastasis, was treated in cohort I with TRT and did not receive prior immunotherapy. The other patient who obtained a CR did not have a brain metastasis, was treated with TIL in cohort II and was progressive after prior immunotherapy including anti-PD-1. The patient who obtained a PR had normal LDH, a confirmed brain metastasis and was immunotherapy naïve before treatment with TRT in cohort II.

Interestingly, 6 out of 14 (42.8%) patients who were not pretreated with checkpoint therapy showed clinical benefit, whereas 4 out of 20 (20%) patients who received prior checkpoint therapy still responded to ACT+IFNa (Figure 1A). In addition, we compared patients who were pretreated with less than three lines of treatment before the start of ACT and IFNa with patients who received three or more prior systemic therapies with respect to their response to ACT and IFNa. Overall, non-responding patients to ACT and IFNa were more frequently pretreated with three or more systemic therapies, when compared with patients who responded, 40% vs 14%, respectively. The 24 non-responder patients showed PD prior, at or after the first evaluation time-point. Interestingly, three out of the seven patients with SD and two patients with an SD <6 months showed a mixed response since some of their lesions clearly showed regression after ACT+IFNa suggesting that the infused TIL did have the capacity to kill tumor cells *in vivo* but that other factors hampered their efficacy in the other lesions (Online Supplementary Figure 3).

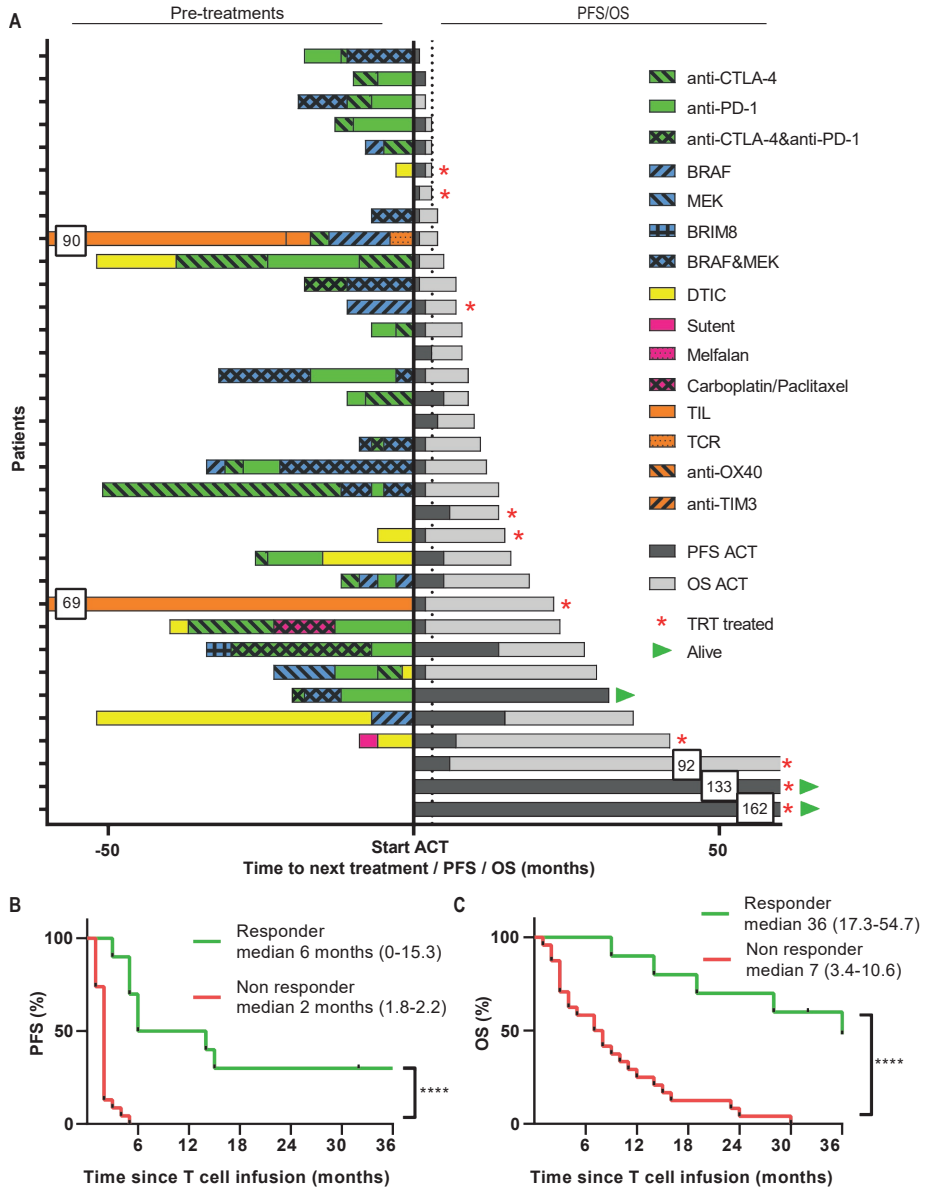

FIGURE 1 Pre-treatment and survival after start of ACT treatment. (**A**) Treatments received before start of ACT are depicted for every individual patient in the left part, followed by their PFS and OS in months in the right part. Kaplan-Meier curves for PFS (**B**) and OS (**C**) as measured from the start of therapy for responding (R, green lines, n=10, defined as CR, PR or SD >6 months) and non-responding (NR, red lines, *n*=24). Differences were calculated using the log rank test, ****$p<0.0001$. ACT, adoptive cell therapy; CR, complete response; OS, overall survival; PFS, progression-free survival; PR, partial response; SD, stable disease.

The responder patients showed a significantly longer time-to-progression when compared with non-responders (Figure 1B). This indicates that the clinical benefit was durable as reflected by the significantly improved 1-year (90.0% versus 28.6%) and 3-year (46.7% versus 0%) OS in the responder and non-responder patients, respectively ($p<0.0001$, Figure 1C). Importantly, interaction analyses between CB and the baseline MLR showed that the difference in time-to-progression after ACT+IFNa between responders and non-responders was not influenced by this baseline characteristic (Online Supplementary Figure 4a). Similarly, the baseline MLR did not influence the OS in responder patients but the effect of the pretreatment MLR on OS was retained in the group of non-responders (Online Supplementary Figure 4b) indicating that ACT+IFNa treatment successfully changed the clinical course of patients, even when they previously had progressed on checkpoint therapy.

IFNa pretreatment induces leukopenia via the reduction of distinct subsets of immune cells

IFNa pretreatment resulted in a mild leukopenia detectable after 1 week of IFNa and characterized by a decrease in total leukocyte, neutrophil, monocyte and lymphocyte counts (Figure 2A-E, pre start of IFNa treatment versus infusion 1). The numbers of leukocytes, in particular neutrophils and monocytes, rapidly bounced back in non-responders. In contrast, in responding patients IFNa pretreatment caused a reduction in leukocytes and neutrophils which was retained during the whole treatment period. No difference was observed in this respect between patients who obtained CR or PR versus SD. The number of these cells were significantly lower than in the non-responding patients at the time of TIL infusions. Monocytes were already lower at baseline and were not altered in responder patients (Figure 2A-E). Consequently, the MLR was always lower in the group of responding patients when compared with the group of non-responders and also did not overtly change over time (Figure 2H). All IFNa-induced changes were transient and recovered within several weeks after cessation of IFNa injections (not shown).

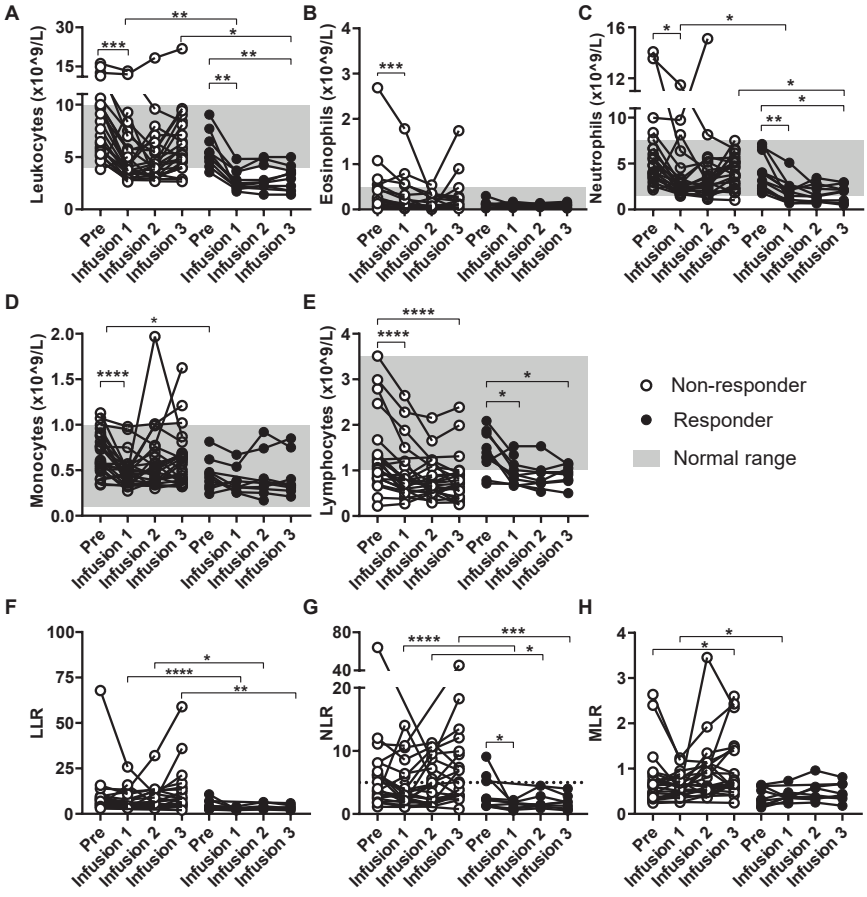

FIGURE 2 Treatment effect on peripheral blood counts. Absolute blood counts were performed on peripheral blood collected at different time points: before start of IFNa treatment (Pre) and at the time of T cell infusions (Infusion 1-3) just prior to the T cell infusion. Data from non-responding patients (*n*=24) are compared with data from responding patients (*n*=10, defined as CR, PR or SD >6 months) in each panel. The absolute leukocyte (**A**), eosinophil (**B**), neutrophil (**C**), monocyte (**D**) and lymphocyte count (**E**) are shown. In addition, the leukocyte-to-lymphocyte (LLR) (**F**), neutrophil-to-lymphocyte (NLR) (**G**) and monocyte-to-lymphocyte (MLR) (**H**) ratios are shown. Differences within patients were calculated using the Wilcoxon signed rank test, data between response groups were calculated using a Mann-Whitney U test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CR, complete response; IFN, interferon; PR, partial response; SD, stable disease.

Leukopenia induced by more intense preconditioning regimens for ACT may result in increased levels of circulating homeostatic cytokines[7]. Therefore, we measured the serum levels of IL-7, IL-15 or IL-21 but no effect of IFNa on these cytokines was observed (Online Supplementary Figure 5).

To study the effects of IFNa on immune cells, the PBMC of 18 patients were analyzed with different sets of antibodies to analyze T cell subsets, MDSC, macrophages and DCs. In general, there were no effects on the percentages of CD3, CD8 and CD4 T cells relative to the total percentage of viable cells (Online Supplementary Figure 6a-c). We used combined Hierarchical Stochastic Neighbor Embedding to analyze the complex set of different T cell populations detected by the antibody mix to inhibitory and memory markers. This revealed three distinct immune populations (clusters), comprising CD8+PD-1+CD45RO+CD62L+CD28+central memory T cells (#1), CD4+PD-1-CTLA-4+TIM-3+CD45RO+CD62L+CD28+central memory T cells (#5) and CD45RO-CD62L+CD28+CD8+PD-1+effector/central memory T cells (#10), which significantly decreased after IFNa pretreatment (Figure 3A-D). Regulatory T cells were gated according to the consensus strategy[25], but no changes were observed (Online Supplementary Figure 6d). Analysis of the different populations of myeloid cells revealed no changes in monocytic MDSC (CD14+HLA-DR-), M1 (CD14+HLA-DR+CD33-CD163-) or M2 (CD14+HLA-DR+CD33-CD163+) blood macrophages or on NK cells (CD3-CD56+) following IFNa treatment (Online Supplementary Figure 6e-h). The percentage of CD14-CD11b-CD11c+DCs, however, decreased (Figure 3E). Identification of the different subsets according to Villani et al[34] showed a decrease in CD32B+DC2, CD141-CD1c-DC4, whereas the CD36+CD163+DC3 and CD123+pDC increased (Online Supplementary Figure 6i-m). Based on the earlier observation that CD14+CD16-HLA-DRhi classical monocytes predicted time-to-progression and OS on PD-1 blockade in metastatic melanoma[35], we analyzed non-classical (CD14±CD16++), CD14+CD16+intermediate and CD14+CD16-classical monocytes[35,36]. Although significant shifts were observed after IFNa pretreatment in the non-classical and intermediate monocytes, this was not the case for the population of classical monocytes (Figure 3F, Online Supplementary Figure 6n,o).

In summary, IFNa pretreatment had distinct effects on different immune cells. Most notably, a sustained reduction in leukocytes and neutrophils was observed during the treatment period in responder patients. This may explain why the number of pre-existent neutrophils was not associated with the time-to-progression after treatment with ACT+IFNa.

ACT products comprise high percentages of CTLA-4 and PD-1 expressing T cells

Ten evaluable patients were treated with TRT and 24 patients received TIL. We previously showed that the TRT in the MLTC cultures of responder patients proliferated stronger than in non-responders[15] and a similar trend was observed here with respect to the TRT and TIL of responders (Figure 4A). The TRT and TIL cultures comprised mainly CD3+ T cells (median and range: 99%, 74%-100%), but varied enormously in the ratio of CD3+CD8+ (median and range: 56.7%, 4%-95%) vs CD3+CD4+ (median and range: 42%, 5%-96%) T cells. Based on the composition of the ACT product, the total number of CD8+ T cells that was infused could be calculated and was shown not to correlate with clinical outcome (Online Supplementary Figure 7a). The majority of the ACT products (MLTC 9 out of 10; TIL 17 out of 24) produced predominantly IFNg when stimulated with the super-antigen SEB (Figure 4B). The ACT product used for treatment of patients who obtained a CR or PR did not differ from other ACT products with respect to proliferation rate, ratio of CD3+CD8+ vs CD3+CD4+ cells or cytokines production.

The expression of the inhibitory markers CTLA-4, PD-1 and TIM-3 was analyzed on 14 ACT products. This revealed that a substantial percentage of the infused T cells express one or more of the checkpoint inhibitory markers (Figure 4C). However, no overt differences were observed between the ACT products given to responder (*n*=6) and non-responder (*n*=8) patients (Online Supplementary Figure 7) Online Supplementary Figure 7. These data suggest that the full capacity of the transfused T cells to control tumor cell growth may have been hampered due to checkpoint inhibition.

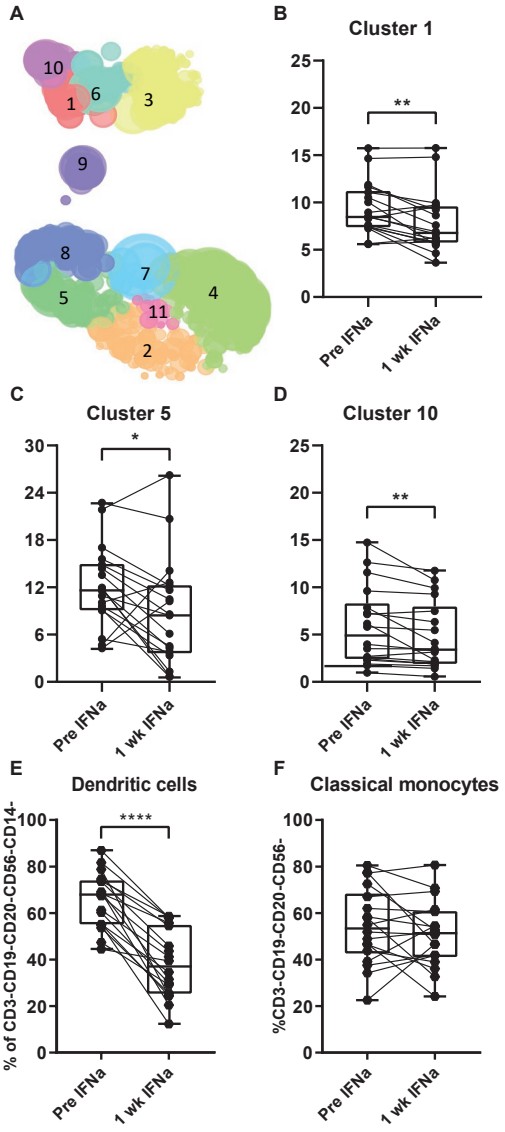

Figure 3 Effect of IFNa conditioning on phenotype of PBMC. Blood was collected before and 1 week after start of IFNa treatment, PBMCs were isolated and phenotypically characterized by flow cytometry. (**A**) The obtained data were analyzed by Hierarchical Stochastic Neighbor Embedding. Paired testing revealed three distinct immune clusters that were significantly decreased in percentage after 1 week of IFNa (**B-D**). Cluster 1 comprises CD8+PD-1+CTLA-4-TIM-3-central memory T cells (**B**), cluster 5 comprises CD4+PD-1-CTLA-4+TIM-3+central memory T cells (**C**) and cluster 10 comprises CD8+PD-1+CTLA-4-TIM-3-effector/central memory T cells (**D**). Significantly decreased percentages of dendritic cells (CD3-CD19-CD20-CD56-CD14-CD11b-CD11c+) (**E**), but not in classical monocytes (CD3-CD19-CD20-CD56-CD14+CD16-) (**F**) are shown. PBMCs from 18 patients were analyzed. Differences within patients were calculated using paired t-test, data between response groups were calculated using an unpaired t-test. Responding patients are defined as having a CR, PR or SD >6 months.
*p<0.05, **p<0.01, ****p<0.0001. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CR, complete response; IFN, interferon; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; PR, partial response; SD, stable disease.

T cell reactivity to private tumor antigens is associated with longer overall survival

An important parameter for ACT is the recognition of tumor cells. As a first screen for T cell reactivity of the ACT products, we stimulated them with an extended panel of 37 different melanoma cell lines and scored the reactivity against all cell lines, matched for at least one HLA class I allele, as already published for a number of the TRT[15]. In 8 of the 21 ACT products tested one or more of the matched cell lines were recognized (Figure 5A-C). Plotting the level of cross-reactivity against OS suggested that treatment with a low (<7%) cross-reactive ACT product often results in longer OS (Figure 5D). The absence of cross-reactivity may also indicate lack of tumor cell-reactivity. In order to elucidate if the correlation between OS and low cross-reactivity reflects the recognition of neoantigens, we set out to identify neoantigen reactivity for the four patients with the longest OS including one CR and one PR patient, of whom also an autologous cell line was available. The presence of neoantigen-specific T cells in the ACT products was previously reported for two of the four patients[26,37] and using the same approach now also in the ACT products of the two other patients with a relatively long survival after therapy. Whole exome sequencing revealed 306 and 605 non-synonymous mutations and based on RNA expression level 207 and 106 potential neoantigens were detected, respectively, in these two patients. Analyses of the peptides harboring the mutated sequences that were recognized showed neo-epitope-specific T cell reactivity against one and seven epitopes, respectively, in each patient (Table 2). These data show that the lack of cross-reactivity in the ACT products of long-living patients more likely is associated with the specific recognition of private antigens.

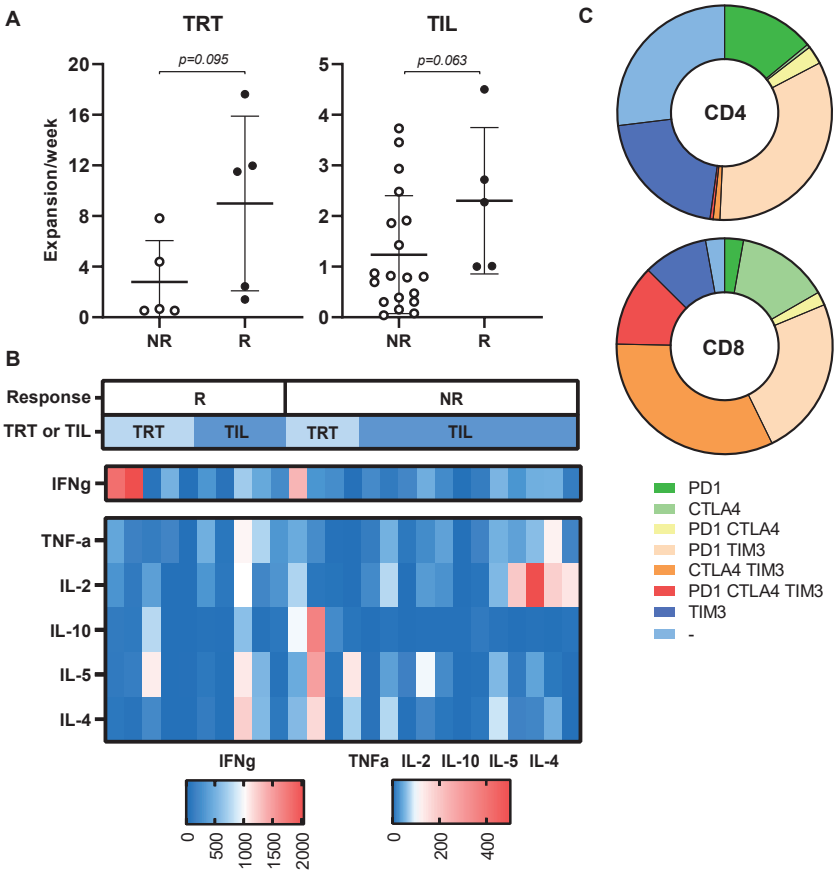

FIGURE 4 Characteristics of the ACT product used for treatment. The expansion rate of TRT (*n*=10) and TIL (*n*=24) used for infusions are depicted for responding (R, defined as CR, PR or SD >6 months) and non-responding (NR) patients (**A**). The expansion rate was calculated as the total number of cells after the initial expansion phase divided by the number of cells (for TRT), or initiated wells (for TIL) at the start of the culture and the duration of the culture period in weeks. Differences were calculated using a Mann-Whitney U test. (**B**) The cytokine profile of the infused ACT products was analyzed after stimulation with staphylococcal enterotoxin B (24 hours) and cytokine production was measured by cytometric bead array assay. The concentration of the indicated cytokines produced by ACT products administered to responding patients (R, left side) and non-responding patients (NR, right side) are shown in the heatmap (*n*=28). Concentrations of cytokines are shown according to the legend boxes below the figure with low concentrations indicated in blue and high concentrations in red. Whether patients were treated with TRT or TIL is indicated in the bar above the figure in light and dark blue, respectively. (**C**) The expression of checkpoint molecules/activation markers was analyzed by flow cytometry on infused T cells. The fraction of negative and single, double or triple positive CD4+ (upper) and CD8+ T cells are depicted in the pie plots (*n*=15). ACT, adoptive cell therapy; CR, complete response; OS, overall survival; PFS, progression-free survival; PR, partial response; SD, stable disease; TIL, tumor infiltrating T cells; TRT, tumor-reactive T cells.

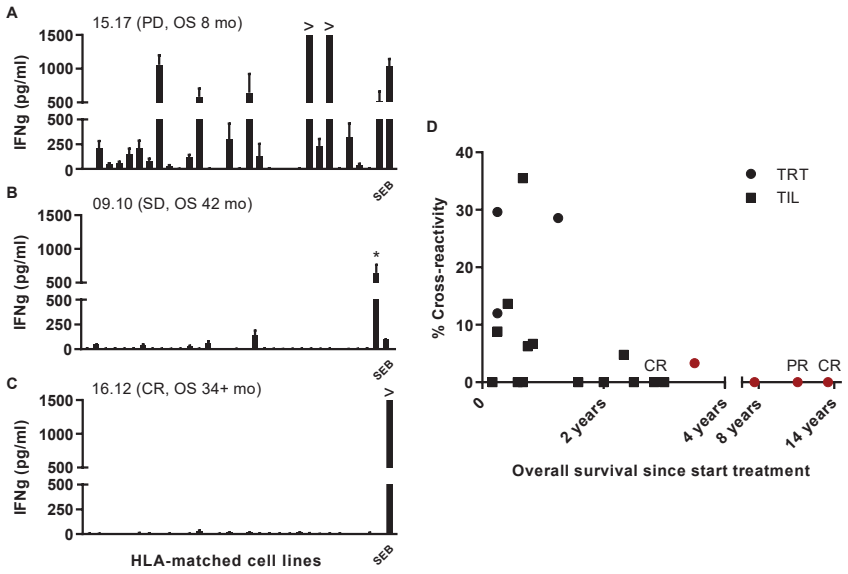

FIGURE 5 Cross-reactivity of ACT products. The recognition of shared antigens was investigated using a panel of melanoma cell lines that share at least one HLA class I allele. Recognition of allogeneic cell lines is defined as cross-reactivity. The percentage cross-reactivity is depicted and calculated by division of number of cell lines recognized by the number of cell lines tested×100%. Results for TRT (*n*=7) and TIL (*n*=14) are depicted. Representative examples of three ACT products with a relatively high or low percentage cross-reactivity are depicted (**A-C**). (**A**) A high percentage cross-reactivity was observed for TIL of patient 15.17, who was progressive on treatment and had an OS of 8 months. (**B**) Shows a rather restricted recognition pattern for TRT of patient 09.10 who obtained stabilization of disease and a relatively long OS, while (**C**) shows the recognition pattern of TIL from a complete responder 16.12, who only recognizes the positive control (SEB). Asterisk (*) indicates the autologous cell line of patient 09.10. ACT, adoptive cell therapy; CR, complete response; HLA, human leukocyte antigen; IFN, interferon; OS, overall survival; PD, progressive disease; PR, partial response; SD, stable disease; SEB, SEB, staphylococcal enterotoxin B; TIL, tumor infiltrating T cells; TRT, tumor-reactive T cells.

Discussion

Adoptive transfer of both TIL and TRT in combination with IFNa is safe, feasible and results in clinical benefit in 10 of 34 (29%) patients with stage IV metastatic melanoma.

The ACT product infused in responders and non-responders did not overtly differ in composition, cytokine production or expression of CTLA-4, PD-1 and TIM-3 co-inhibitory molecules. However, we observed in a number of cases cross-reactivity to melanoma cell lines which were HLA-matched for at least one allele. In those cases, the patients displayed short OS after treatment, while a longer OS was observed for the patients of which the ACT product showed no to low cross-reactivity to allogeneic

HLA-matched melanoma cell lines. Four of the very long survivors were treated with an ACT product that displayed no cross-reactivity, but recognized somatically mutated antigens identified in the autologous melanomas. This suggests that treatment with neoantigen-specific T cells may increase clinical benefit. This is supported by the finding that mutational load predicts clinical outcome after ACT in patients with melanoma[38] and that response to checkpoint inhibitors mediated by reinvigoration of tumor-specific T cell reactivity is also correlated with mutational load in melanoma and other malignancies[39-41].

Similar to what has been reported for other immunotherapy trials[30-33], the MLR was associated with shorter OS of the whole group by multivariate analyses. Elevated neutrophil-to-lymphocyte ratio (NLR) has been shown to predict poor response to nivolumab in melanoma[42]. There was no apparent association between NLR and OS in our trial, which may be explained by the fact that the NLR is normalized by IFNa conditioning thus abrogating impact on survival. In contrast to the NLR, the MLR was associated with shorter survival and shorter time-to-progression. Interestingly, the MLR displayed an impact on OS only in those patients who did not respond to therapy, as shown in the interaction analyses. Apparently, the MLR normalization by IFNa, which was most pronounced in the non-responding patients displaying higher pretreatment MLR levels, was not strong enough to revert the impact of baseline levels on OS and progression. The relatively mild leukopenia obtained by IFNa may also explain why we do not observe an increase in homeostatic cytokine levels. Elevation of serum IL-7 and IL-15 levels after lymphodepletion are suggested to be of critical importance for clinical response after ACT[7], although elevated levels were not directly compared with clinical response and especially the role of IL-7 seems less important[22,43]. Nevertheless, if these cytokines and induction of leukopenia are of major importance for treatment outcome it is advised to choose a more intense conditioning regimen for patients with a relatively good condition, whereas the mild conditioning using IFNa may be more appropriate for the remaining patients otherwise not eligible for ACT.

Overall, IFNa conditioning induces leukopenia and neutropenia and favorable blood count ratios that, if persistent during therapy, correlate with clinical response. Leukocytosis has been suggested to be driven by the increased production of homeostatic cytokines, in particular granulocyte-colony stimulating factor and IL-6, by tumor cells or other cells in the tumor micro-environment[44,45], which augments hematopoiesis and migration of myeloid progenitor cells from the bone marrow to the blood. Potentially, the infused T cells of responder patients effectively reduced the tumor load, thereby decreasing the production of homeostatic cytokines and consequently the induction of leukocytosis. Hence, a failure of the ACT product to control tumor growth may explain the leukocyte rebound as observed in non-responders.

We observed that the percentage of immunotherapy-naïve patients responding to therapy is twice that of the group of patients who were progressive on prior immunotherapy, confirming the results of a recently published study in which patients progressive on CTLA-4 blockade responded worse to ACT than CTLA-4-naïve patients[11]. More importantly, our data show that patients with resistance to PD-1 blockade may still respond to ACT using a mild conditioning and support regimen, confirming other recent studies reporting a 22%-38% response rate after ACT in patients resistant to anti-PD-1 immunotherapy[22,46]. Interestingly, Sarnaik et al. reported that TIL therapy was not effective in patients who developed secondary resistance to PD-1 blockade[46]. These findings underscore the hypothesis that patients who acquire immune escaped tumor variants after checkpoint blocking therapy may include modifications that also affect TIL-mediated tumor eradication, for example, antigen loss or HLA loss or other defects in the antigen processing pathway[47]. However, some of the patients in our study developing SD after ACT included a patient who initially had responded to anti-PD-1, indicating that secondary resistance to checkpoint therapy does not exclude patients to benefit from ACT therapy per se. This latter also applies to patients who develop (severe) autoimmune side effects leading to permanent discontinuation of checkpoint blockade, which occurs in approximately 15% of the cases[48]. Patients achieving CB in our trial displayed a lower objective response rate when compared with a recently reported ACT trial in patients with melanoma[11]. This may partially be due to the fact that a higher number of patients in our trial had unfavorable staging and LDH levels, and also received more lines of prior therapy. The fraction of patients in our trial that were pretreated with anti-CTLA-4 (with or without anti-PD-1) was twice as high as that in the study by Forget et al.[11]. In their study, this was shown to result in reduced response to therapy and shorter OS compared with that obtained in treatment-naïve patients (24.6 versus 8.6 months; HR, 2.3; 95% CI, 1.3 to 4.1, p=0.003). However, the median OS in the CTLA-4-pretreated group in their trial (8.6 months) was similar to what was observed in our trial (9 months).

A substantial percentage of the infused T cells express one or more of the inhibitory checkpoint molecules CTLA-4, PD-1 or TIM-3. Whereas the transient expression of PD-1 and other checkpoint molecules is induced after normal T cell activation, the sustained expression and gradual accumulation of multiple checkpoint molecules is associated with T cell exhaustion due to continued antigenic stimulation in the tumor environment comparable to what is observed during chronic viral infection. Continued expression of multiple checkpoint molecules is associated with gradual loss of effector function and proliferative capacity[49]. The association between impaired proliferation of infused T cells with worse clinical response observed in our trial, thus may reflect an increased exhausted phenotype, although there is no significant difference in the frequency of inhibitory marker positive T cells between infusion products administered to responding and non-responding patients.

However, the simultaneous expression of multiple inhibitory checkpoint molecules may reflect true exhausted T cells[50-52]. To overcome this, ACT in combination with anti-PD-1 is proposed and implemented in our recently initiated and currently ongoing trial (NCT03638375).

TABLE 2 Mutation load, putative and identified immunogenic neoantigens in melanoma cell lines.

Cell line Code (response)	InDELs	Substitutions (total)	Non-synonymous substitutions	Tested peptides	Recognized T cell epitopes	Reference
MEL 04.01 (SD >6 months)	3	487	320	226 SLP	EML1(R64W) SEPT2(R300C) CAD(R1854Q)3	26
MEL 05.18 (CR)	1	1243	811	501 SLP	RPS12(V104I) ZC3H18(G269R) TNIK(S502F) KIAA0020(P451L) ribosomal protein RPL28(S76F)	39 39 39 26 26
MEL 08.11 (PR)	0	442	306	207 SLP	TP53 (L194F)	This article
MEL 09.10 (SD >6 months)	2	952	635	106 SSP	CLPTM1 (P485L) ETV5 (P465S) NIPAL2 (L95P) TNFRSF12A (I197N) MPDU1 (P213L) ERRFI1 (L338F) ZNF532(S263L)	This article This article This article This article This article This article This article

The mutation load, defined by number of insertions and deletions (InDELs) and the total number of substitutions is depicted. The number of synthetic long (SLP) or short (SSP) peptides comprise all the non-synonymous substitutions with have a detectable RNA expression (>0) in the tumor sample, excluding those that introduce a premature stop-codon. CR, complete response; PR, partial response; SD, stable disease.

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Contributors

EV, EK and SvdB designed the clinical study. GL performed surgery and provided the resected tumor. PM and ATvS performed QP and QC checks of the production process and of the infusion product. IR and EK treated the patients.

EV, MvdK, EK and SvdB analyzed and interpreted the patient data. EV, MvdK and SvdB designed and interpreted the monitoring experiments. EV, MvdK, MV, CvdM, LdB produced the infusion product and performed all analysis. MW and SS designed and validated the Ab panels for flow cytometry. EV, NdM, MV and SvdB designed, executed and interpreted the neoantigen identification experiments. EV, MvdK and SvdB wrote the manuscript and all authors read and approved the final manuscript.

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Competing interests

None declared.

Patient consent for publication

All patients gave written informed consent before inclusion in this study.

Ethics approval

This phase I/II study was approved by the Medical Ethics Committee of the Leiden University Medical Center (study number P04.085) and conducted in accordance with the Declaration of Helsinki.

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CHAPTER
9

Adoptive cell therapy in metastatic melanoma

CHAPTER
9.1

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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Monique K. van der Kooij, Els M.E. Verdegaal, Marten Visser, Linda de Bruin, Caroline E. van der Minne, Pauline M. Meij, Inge C.F.M Roozen, Mare A. Jonker, Shelley van den Bosch, Gerrit-Jan Liefers, Frank M. Speetjens, Sjoerd H. van der Burg, Ellen Kapiteijn

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[1]. The presence of sufficient numbers of activated T cells is a requirement for a durable response to anti-PD-1[2]. 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[3-6]. 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[7,8]. 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)[7].

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.

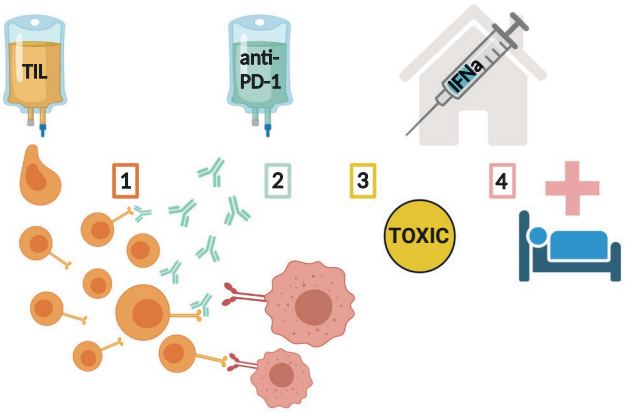

FIGURE 1 Resolving four of the most important aspects curtailing the efficacy and feasibility of current immunotherapies: (**1**) providing tumor-reactive TIL; (**2**) alleviating immune checkpoint inhibition; (**3**) reducing toxicity of ACT treatment; (**4**) Minimalizing hospitalization and patient burden. ACT, adoptive cell therapy; IFNa, 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[2]. 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[9]. 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[7]. 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[10] need to be resolved to push ACT more to the forefront of melanoma care[11,12]. 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[13-15]. 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[3,4,16,17].

IFNa has been shown to result in a discernible but mild and transient leucopoenia[7,8,18,19] and is routinely used in allogeneic stem cell transplantation to support donor lymphocyte infusions[20]. 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[7,8].

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-IFNa, 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)[21,22]. 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
- ≥18 years old and histologically proven unresectable (or residual) regional metastatic cutaneous melanoma.
- Eastern Cooperative Oncology Group (ECOG) performance status ≤1.
- Treated with standard treatment options (anti-PD-1, cytotoxic T-lymphocyte-associated protein 4 antibody, ±BRAF/MEK-inhibition) and experiencing progressive disease according to RECIST 1.1.
- Within 2 weeks prior to study: hemoglobin ≥6.0 mmol/L, creatinine clearance ≥60 min/mL, aspartate transaminase and alanine aminotransferase ≤5× the normal upper limit, lactate dehydrogenase ≤2× 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 (≥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.

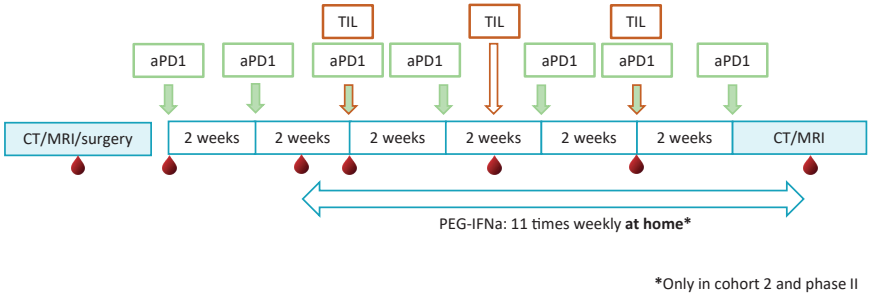

FIGURE 2 Study design of ACTME trial. Blood and serum are collected at indicated time-points (red blood drop). In cohort 1, treatment with PEG-IFNa is omitted. In cohort 2 and phase II, pegylated-IFNa is added to the treatment with aPD1 and TIL. aPD1, anti-PD-1; IFNa, interferon-alpha; PEG-IFNa, pegylated-interferon-alpha; 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[7,8]. We use a fixed 4-week TIL culturing period. Furthermore, based on our previous findings, we implemented a TIL dose range of 2.5-7.5×10^8 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 (1-2.5×10^8, 2.5-5 ×10^8 and 7.5-10×10^8) in our previous study[7]. 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-IFNa 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-IFNa.

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[23]. 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 [7,24,25].

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[7,8,26]. The supernatants of these tumor stimulated TIL cultures are used to determine specific production of IFN-γ, TNFα, 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[7,8,25].

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[4]. The effect of PEG-IFNa 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).

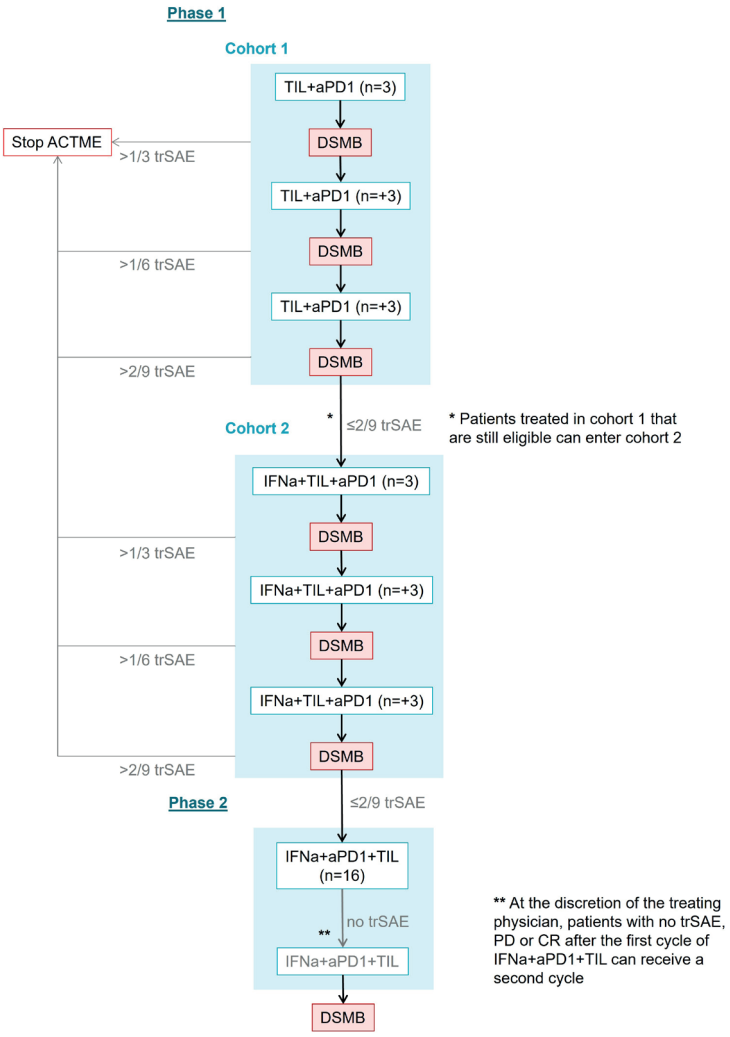

FIGURE 3 Number of patients treated per cohort and in the two study phases and data safety monitoring during ACTME trial. aPD1, anti-PD-1 treatment; DSMB; Data Safety Monitoring Board; IFNa, peginterferon-alpha2a; TIL, tumor infiltrating lymphocytes; trSAE, treatment-related serious adverse event.

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[27].

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[28,29]. Patients eligible for this phase I/II clinical trial are refractory to the standard treatment lines. Therefore, a response rate of less than 10% (P0) would not be sufficiently large enough to warrant further investigation. A response rate of 30% (P1) 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 α of 5% and 80% power (β), this requires a total of 25 patients in our study (α=0.05, β=0.20, P0=10%, P1=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 α of 5% and 80% power (β), 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[30].

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[1]	X			X	X		X		X			X
Physical examination[2]	X			X	X		X		X			X
Viral serology and HLA typing[3]	X											
Pregnancy test (females)[4]	X											
CBC differential + Blood chemistry[5]	X			X	X		X		X			X
Tumor metastasectomy[6]	X											
Nivolumab i.v.		X	X		X	X		X	X	X		
PEG-IFNa s.c.[7]				X	X	X	X	X	X	X	X	
TIL infusion8					X		X		X			
Imaging studies[9]		X									X	
Extra blood sample[10]	X	X		X	X		X		X			X
Plasma collection[11]	X	X		X	X		X		X			X
Biopsy metastasis[12]												X

1 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 be performed as part of the standard care for melanoma patients.
2 Complete physical examination.
3 Viral serology and HLA typing: HBsAg, IgG anti-HBc, IgG anti-HCV, IgG anti-HIV1/2, HIV Ag, IgG anti-HTLV, TPHA.
4 For female patients of child bearing age only.
5 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 T4, TSH and cortisol. Complete Blood Count includes: white blood cell count and differentiation, red blood cell count, hemoglobin, MCV, platelet count.
6 Tumor tissue is resected and used to culture TILs.
7 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).
8 Tumor Infiltrating Lymphocytes (TIL) are administered.
9 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.
10 For analysis of treatment effect on immune parameters 50 ml of heparinized venous blood is obtained.
11 Plasma is be collected from the blood drawn for point 10 and kept stored at -20°C.
12 Biopsy of a second metastasis (if feasible) is performed after the first TIL-infusion cycle for additional molecular biological and immunological tests.

CHAPTER
9.2

Preliminary results phase I of the ACTME trial

Results

Patients and treatment

Between November 2018 and September 2019 all nine patients from cohort 1 started their treatment in the ACTME trial. Between January 2020 and May 2021 the nine patients in cohort 2 all started their treatment. In the first cohort, nine patients were treated with ACT and anti-PD-1. After every group of three patients, the data was presented to the DSMB. They concluded that the combination treatment of cohort 1 was safe and supported to start cohort 2. Hereafter, nine patients were treated with the final combination of ACT, anti-PD-1 and PEG-IFNa. Again after treating every 3 patients, the safety data was presented to the DSMB.

The baseline characteristics of all 18 patients are shown in Table 1. Four patients who signed the Patient Information Folder were eventually unable to start the trial due to fast progressive disease, and are therefore not included in Table 1. In one of these patients the fast disease progression followed after cessation of BRAF/MEK inhibition, and one patient already had fast disease progression during treatment with BRAF/MEK inhibition. Of the four excluded patients, three were men, the mean age was 62.8 years. The majority of the evaluable patients treated in the trial were men (72.5%), the mean age was 53.5 years, and one-third of patients had brain metastases at the time of inclusion. According to the inclusion criteria, all patients had progressed on anti-CTLA-4 and anti-PD-1 treatment.

Safety

None of the treated patients experienced TIL-related adverse events (Table 2). Seven patients (38.8%) experienced grade 1 adverse events, two patients (11.1%) had grade 2 adverse events, and one patient (5.5%) suffered from grade 3 diarrhea. All other 7 patients (38.8%) did not report any adverse events.

TABLE 1 Patient characteristics at baseline

Patient	Cohort	Age	Gender	WHO	LDH	CNS metastasis	Pre-treatments	Location metastasis	TIL culture location
2	1	48	Male	0	158	No	BRAF/MEK, aPD1, aCTLA4	LN	LN
4	1	47	Female	1	270	No	BRAF/MEK, aPD1&aCTLA4, TIL	Bone, lung, muscle, adrenal, liver	Bone (sternum)
5	1	54	Male	0	230	Yes	aCTLA4, aPD1	LN, lung	LN
6	1	60	Male	1	224	No	aPD1, aCTLA4, aPD1	Muscle, lung, liver, peritoneum	Liver
7	1	53	Female	0	197	No	aPD1, BRAF/MEK, aCTLA4	LN, liver	Liver
8	1	40	Male	0	217	Yes	aPD1&aCTLA4, BRAF/MEK, aPD1	Subcutaneous, Liver, gall bladder, cardiac	Subcutaneous
9	1	53	Male	1	241	No	aPD1, aCTLA4	LN, subcutaneous, lung, liver kidney, GI/peritoneum	Subcutaneous
10	1	75	Male	1	258	No	aCTLA4, aPD1	LN, liver	Liver
11	1	53	Male	0	220	No	aPD1&aCTLA4, BRAF/MEK	LN, liver, lung	LN
12	2	26	Female	1	243	No	aPD1, BRAF/MEK, aCTLA4	LN, fatty tissue leg,	Cutaneous
13	2	73	Female	0	374	Yes	anti-PD1, anti-CTLA4	LN, spleen, lung, pleura, brain	LN
16	2	42	Male	0	314	Yes	Dendr cells, aPD1, aCTLA4, aAXL	LN, spleen	LN
18	2	50	Male	0	356	No	aPD1, aCTLA4	LN, liver	liver
19	2	39	male	1	403	No	aCTLA4, aPD1, aPD1&aCTLA4	LN, bone, lung	bone
20	2	75	male	1	210	Yes	aPD1, aCTLA4	LN, adrenal gland, fatty tissue	LN
21	2	49	Male	1	174	No	aPD1, aCTLA4	Parotid gland, LN, (sub)cutaneous	LN
22	2	62	Male	1	201	No	aPD1, aCTLA4, ACTME	LN, lung, liver, muscle	Subcutaneous
23	2	64	Female	1	313	Yes	aPD1&aCTLA4, aPD1, imatinib	LN, adrenal gland, lung, omentum, bone	LN

WHO: World Health Organisation Performance Status, LDH: Lactate dehydrogenase, aPD1: anti-PD-1, aCTLA4: anti-CTLA-4, dendr cells: dendritic cell therapy, aAXL: anti-AXL, LN: lymph node, TIL: Tumour Infiltrating Lymphocytes

TABLE 2 Response to treatment

Patient	TIL-related toxicity (S)AE	Any treatment related (S)AE	Best overall response	Duration of response
2	None	None	PD (MR)	n.a.
4	None	None	PD (MR)	n.a.
5	None	Grade 1 headache	PD	n.a.
6	None	Grade 1 headache, grade 1 hypertension	**PR**	22 months
7	None	None	PD	n.a.
8	None	None	PD (MR)	n.a.
9	None	None	PD (MR)	n.a.
10	None	Grade 3 diarrhea	PD (MR)	n.a.
11	None	None	PD (MR)	n.a.
12	None	Grade 1 fever	PD	n.a.
13	None	None	**SD**	9 months
16	None	Grade 1 rash, grade 1 leukopenia	PD (**irRECIST: SD**)	n.a.
18	None	Grade 1 itch, grade 1 fatigue	**SD**	16 months
19	None	Grade 2 diarrhea, grade 1 rash, grade 1 leukopenia	PD	n.a.
20	None	Grade 1 Lymphopenia, grade 1 itch, grade 1 lethargy	PD	n.a.
21	None	Grade 1 thrombocytopenia, grade 1 itch	**ongoing PR**	11 months+
22	None	Grade 2 hepatitis, grade 1 fever, grade 1 fatigue	PD	n.a.
23	None	Grade 2 anemia	PD	n.a.

TIL: Tumor Infiltrating Lymphocytes, (S)AE: (serious) adverse event, PD: progressive disease, MR: mixed response, PR: partial response, SD: stable disease, irRECIST: immune-related response evaluation criteria in solid tumors, n.a.: not applicable

Clinical responses

In total, disease control was observed in 5 out of 18 patients (27,8%). In cohort 1, one out of nine patients (11,1%) responded and obtained a partial response. In cohort 2, four out of nine patients (44,4%) responded; two patients obtained a stable diseases and one a partial response according to RECIST1.1. In addition, one patient obtained a SD according to immune-related response criteria (irRC) (Table 2, Figure 1). The duration of the responses is shown in Table 2 and Figure 1. In Figure 1 also the duration of response to the previous treatments is depicted. Interestingly, patient 6, who had a partial response to treatment in cohort 1, initially responded but developed resistance to treatment with anti-PD-1 just before inclusion in our trial, while patient 21 with an ongoing partial response to the treatment in cohort 2, displayed primary resistance to previous treatments with anti-CTLA-4 and anti-PD-1 (Figure 1).

The size of the target lesions in cohort 1 (Figure 3) and cohort 2 (Figure 4) was followed in time. In patient 6 a long lasting partial response was observed. In patient 8 a relatively large metastasis disappeared over the course of the first treatment cycle. Multiple patients (# 2, 4, 8, 9, 10, 11) display some form of mixed response, as some lesions become smaller, while others grow (Figure 3).

The same pattern can be seen in the patients in cohort 2 (Figure 4). There, patient 21 has an ongoing partial response, and patient 13 had stable disease of the target lesions but was eventually defined as progressive because a new lesion appeared. Patient 16 displayed a mixed response when the target lesions were considered, but also developed new lesions resulting in progressive disease.

Translational studies

Although most translational studies including immunohistochemistry and serum/plasma marker tests are still ongoing, an initial test already showed that patient 13 with a stable disease for 9 months following treatment in cohort 2 still had an HLA class I proficient tumor, while non-responders 2, 5, and 7 all had lost their HLA class I before inclusion in the ACTME trial (Figure 1).

In contrast to what might have been expected based on our previous trial[1], only a trend in total leukocyte and neutrophil count reduction was observed in patients treated with anti-PD-1 and TIL in combination with PEG-IFNa in cohort 2. Monocyte (not shown) and lymphocyte counts as measured in the peripheral blood were not affected. Due to the small number of patients, it is not possible to draw any conclusions on the difference in peripheral blood count cell subtypes between patients with or without a clinical response (Figure 2).

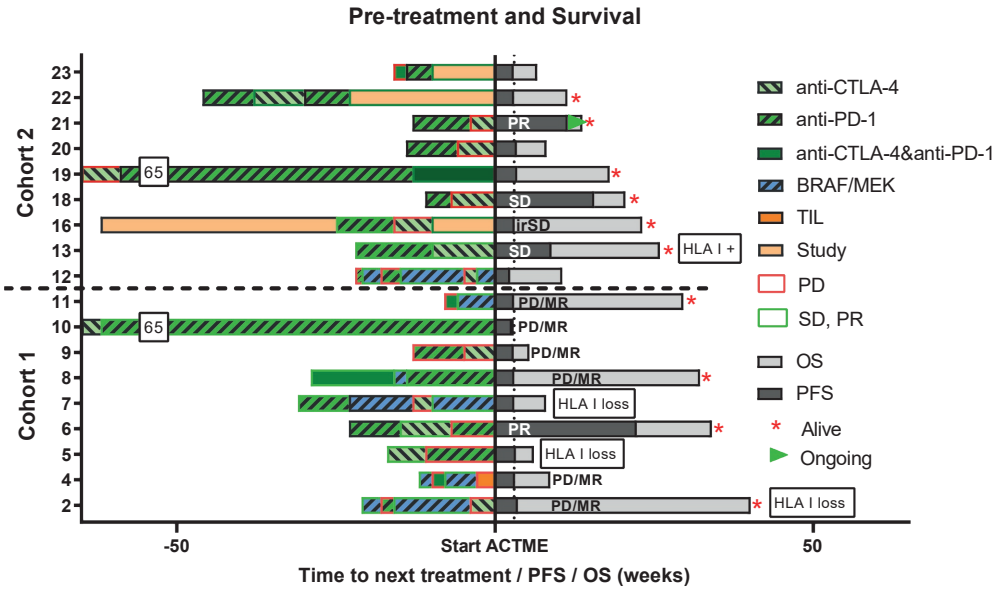

FIGURE 1 Duration and response to pre-treatment, and survival following treatment in ACTME. Treatments received before start of treatment in ACTME trial are depicted for every individual patient in the left part, followed by their Progression Free Survival (PFS, dark grey) and Overall Survival (OS, light grey) in weeks in the right part. Patients below the dotted line received Tumor Infiltrating Lymphocytes (TIL) and anti-PD-1 (cohort 1), while patients depicted above the dotted line received TIL, anti-PD-1 and pegylated-interferon-alpha (PEG-IFNa) (cohort 2). The response according to RECIST 1.1 is shown for responding patients; partial response (PR) or stable disease (SD). One patient in cohort 2 had an immune-related stable disease (irSD) according to the immune-related response criteria. Several patients with progressive disease (PR) had a mixed response (MR), where at least one tumor lesion was reduced in size. Human leukocyte antigen type I (HLA type I) genotyping was performed on patient's PBMC followed by flowcytometric evaluation of the surface expression on the tumor cell lines using specific antibodies. Cell lines were either HLA type I proficient (HLA I +), or HLA deficient (HLA I loss).

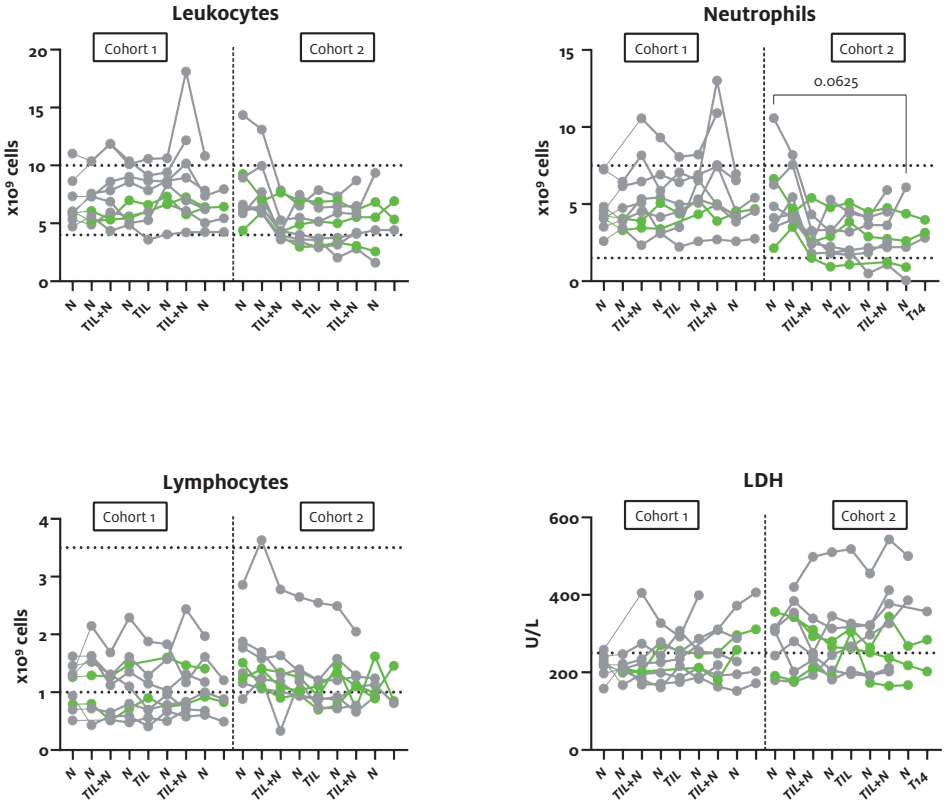

FIGURE 2 Treatment effect on peripheral blood counts.

Absolute blood counts and LDH plasma concentrations were determined in peripheral blood collected at different time points: before start of the first and every subsequent gift of Nivolumab (N), and at the moment just before each infusion of Tumor Infiltrating Lymphocytes (TIL).
In green the values of patients with a clinical response are shown.
Differences within patients were calculated using the Wilcoxon signed rank test, data differences between response groups were calculated using a Mann-Whitney U test.

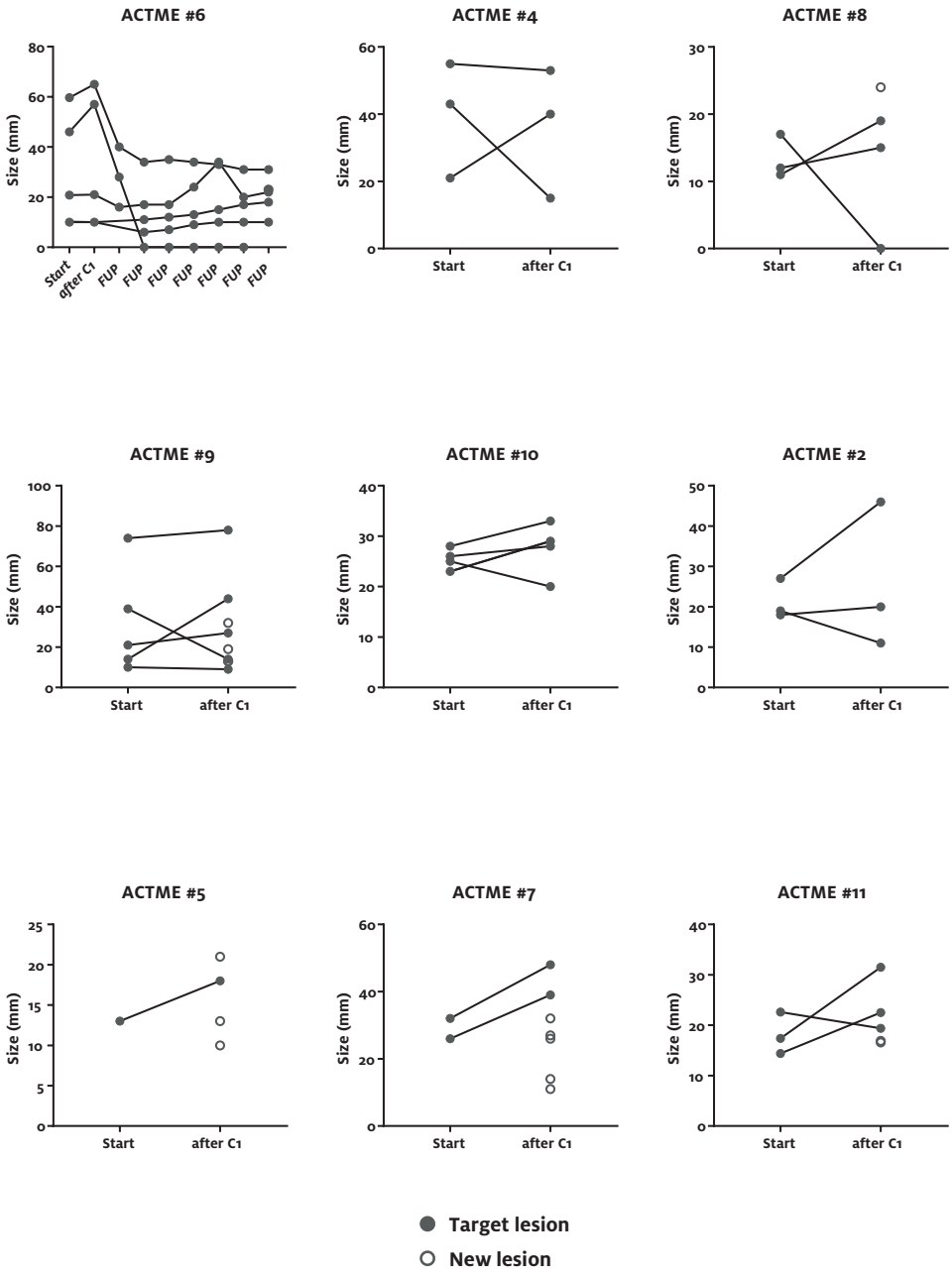

FIGURE 3 Change in lesion sizes in patients treated in cohort 1. The target lesion sizes of individual patients treated in cohort 1 are shown prior to (start) and after TIL infusions (C1). The best overall response of patients #6 is partial response, patients #2, 4, 8, 9, 10, and 11 have progressive disease with a mixed response according to RECIST1.1. In patient #8 one target lesion even disappears under treatment.

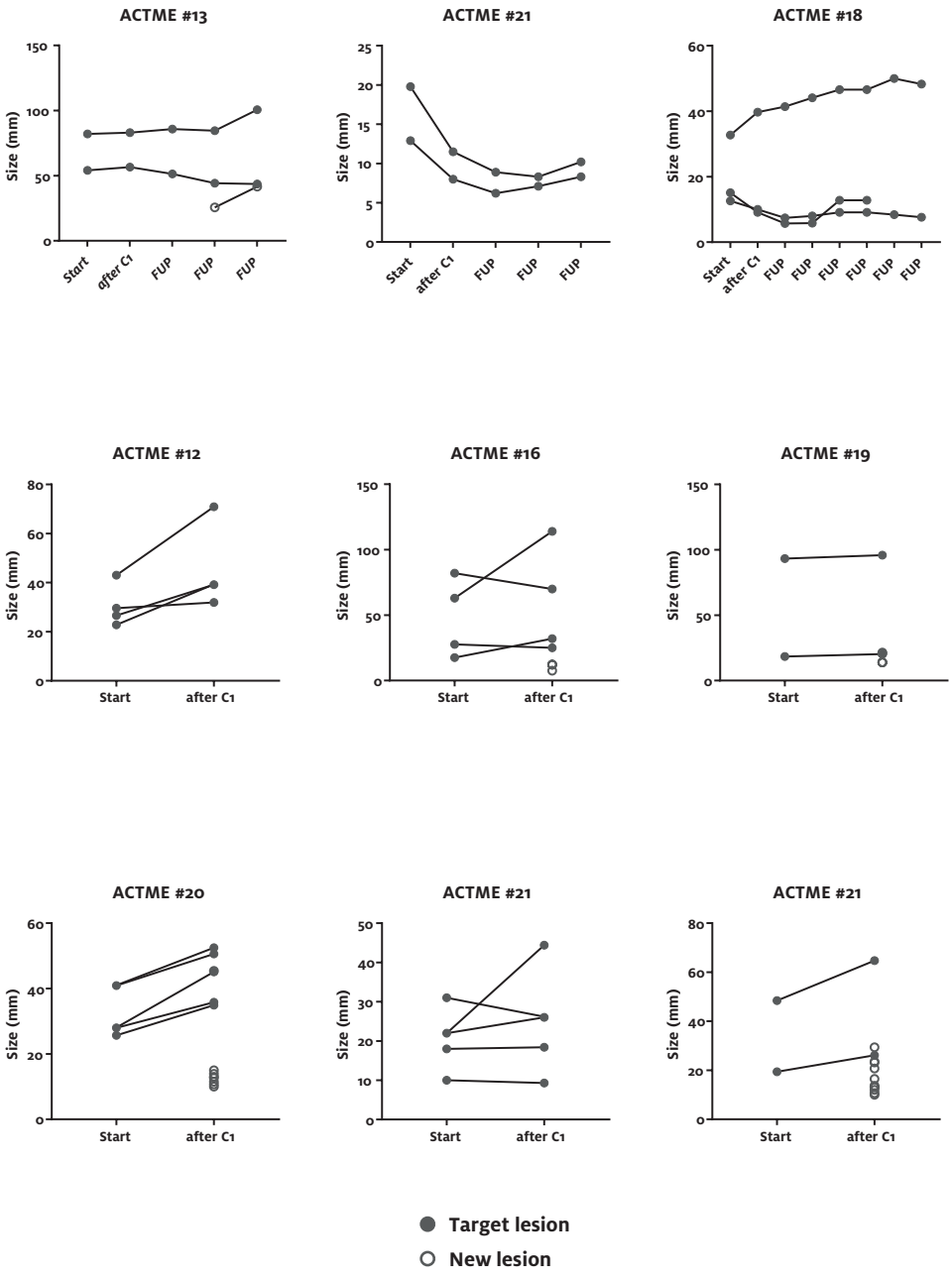

FIGURE 4 Change in lesion sizes in patients treated in cohort 2. The target lesion sizes of individual patients treated in cohort 2 are shown prior to (start) and after TIL infusions (C1). The best overall response of patients #13 and #18 is stable disease, patient #21 has a partial response according to RECIST1.1 and patient #16 has stable disease according to irRECIST.

Discussion

These preliminary data of phase I of the ACTME trial show that the combination of ACT, anti-PD-1 and PEG-IFNa can be safely given to patients with metastatic melanoma, and causes relatively few (serious) adverse events.

At the time of writing the phase II part of the ACTME trial is still ongoing, this will evaluate the efficacy of the treatment combination. So far, the phase I part already gives an indication that the treatment is safe and can result in clinical responses in patients with metastatic melanoma refractory to standard immunotherapy options.

A possible explanation for the difference in treatment effect on peripheral blood counts when comparing our current data with the data from our previous phase I/II trial with IFNa and TIL, is the fact that in the ACTME trial PEG-IFNa is used. Leukopenia has more frequently been reported as a side-effect of IFNa (>10%) when compared to PEG-IFNa (incidental)[2]. However, no head-to-head comparison has been made so far. Although we have previously shown that leukopenia is correlated with clinical response[1], it remains challenging to see if this phenomenon is crucial for the clinical outcome since in the currently ongoing trial clinical responses were obtained in patients who did not experience such leukopenia.

An additional objective of the study is to investigate if some markers in either the infusion product, serum or tumor of the treated patients correlate with clinical results. In this respect, our preliminary data showed that 3 non-responders had HLA I deficient tumors, while the tumor of 1 responder was HLA I proficient. HLA type I loss is described as a very effective immune evasion mechanism of tumor cells[3-9] and may be triggered by T cell mediated therapy including our combination treatment, thus explaining the unresponsiveness to treatment. If HLA class I expression is already absent before treatment, this will hamper further effectiveness of treatment relying on reinforcement of anti-tumor T cell immunity.

Therefore, examination of the HLA class I expression on tumors in the additional patients treated in our trial will reveal whether HLA type I deficiency should be added as an exclusion criteria.

So far, we do not know exactly why some lesions within one patient do respond to treatment while others do not (intra-patient heterogeneity). It is possible that certain tumor characteristics, like the already mentioned HLA class I loss or the presence of specific mutation-derived neoantigens, are not present in all metastases. Additionally, (stromal) immunosuppressive factors could vary depending on the location and the perfusion of the specific tumor.

Further research will be needed to study the influence of the characteristics of the infusion product on the response to treatment. This includes phenotype of the T cells, and the effect of these markers on the persistence of the T cells. Furthermore, the specificity of the T cell product will have to be studied, including the broadness of the tumor-reactivity. As a broad tumor-reactivity could less easily result in the development of antigen escape variants of the tumor, it would be interesting to see whether mixing T cells from multiple lesions of one patient will lead to a better and longer lasting tumor control.

The fact that patients with primary resistance to anti-CTLA-4 and anti-PD-1 immunotherapy can still respond to our new treatment combination is interesting and suggests that a lack of sufficient numbers of tumor-reactive T cells is one of the underlying mechanisms hampering the effect of the checkpoint inhibitors. Potentially, the combined ACT treatment may overcome this by providing the required numbers of tumor-specific T cells that are subsequently unleashed by anti-PD-1 to lyse the tumor cells.

In conclusion, these promising preliminary data warrant full evaluation of the safety and clinical efficacy of the combination treatment after completion of phase II of the ACTME trial.

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CHAPTER
10

General discussion and future perspectives

In this thesis I have been crossing borders in the field of melanoma research, including uveal versus cutaneous melanoma, the use of real-world data to assess safety and efficacy of immune checkpoint inhibition treatment, and the use of adoptive cell therapy in cutaneous melanoma. Now it is time to focus on the horizon.

Part I: Systemic therapies for uveal melanoma

As **Chapter 1** already summarized, many new treatment options have become available for patients with cutaneous melanoma. Fortunately, several of these new therapies are also studied in patients with uveal melanoma, as the treatment options for this group of patients is still limited. **Chapter 2** of this thesis gave us an overview of some of the differences between cutaneous and uveal melanoma. One of the most striking differences in the metastatic setting is the lower mean somatic mutation rate in uveal melanoma, and therefore the potential lack of neoantigens to be recognized by the patient's immune system. This could be one of the reasons for the very limited effect of immune checkpoint inhibition treatment in these patients with either anti-CTLA-4 (**Chapter 3.1**), and anti-PD-1 (**Chapter 3.2**).

In a retrospective analysis 2 of the 6 uveal melanoma patients had a partial response to treatment with the combination of both anti-CTLA-4 and anti-PD-1. Both patients had received a liver metastases-directed therapy before the start of immune checkpoint inhibition[1]. One of these liver-directed therapies is isolated hepatic perfusion (IHP). The principle of IHP is to temporarily isolate the liver from the systemic circulation in a surgical procedure. Subsequently, the liver is flushed with high-dose melphalan (chemotherapy) for an hour. This leads to a local high dose intensity, which would be toxic and induce complications and serious adverse events when administered systemically. However, as the (surgical) procedure is associated with morbidity and even mortality, a new procedure was developed in which hepatic infusion with simultaneous chemofiltration can be performed percutaneously[2-9]. Percutaneous hepatic perfusion (PHP) is a relatively novel alternative to IHP that enables vascular isolation and perfusion of the liver by using endovascular techniques. Important advantages of PHP over IHP are the minimal invasiveness and the repeatability[10]. As metastatic uveal melanoma is associated with isolated diffuse hepatic disease (**Chapter 2** and **4**) PHP has gained popularity over the past two decades. Returning to the cancer-immunity cycle in Figure 2 of **Chapter 1**, we see that PHP treatment with high-dose melphalan could lead to the release of cancer cell antigens, which may be ingested and processed by antigen presenting cells for subsequent presentation to T cells. In 26 patients with advanced cutaneous melanoma, the combination of isolated limb infusion with melphalan followed by systemic administration of anti-CTLA-4 led to a response rate in 85% of patients[11]. Combining the locally administered melphalan by PHP with systemic treatment with immune checkpoint inhibition could as such

also induce a systemic effect by stimulating the endogenously activated T cells in uveal melanoma. A phase 1b/2 study combining hepatic percutaneous perfusion with anti-CTLA-4 and anti-PD-1 in advanced uveal melanoma is ongoing in the Leiden University Medical Center (NCT04283890)[12]. Similar trials are ongoing in other tumor types where responses to monotherapy with immune checkpoint inhibitors (ICI) are rare, including myxofibrosarcoma[13] (NCT04332874). The potential synergistic effects of these combinations will hopefully lead to new standard of care treatment options for patients with these (rare) tumor types.

It was previously shown that long-term survival and clinical benefit from adoptive cell therapy in cutaneous melanoma was determined by a four-parameter tumor immune signature; more CD8 T cells, a high M1/M2 macrophage ratio, more galectin-9 dendritic cells, and the expression of galectin-3 by tumor cells[14].

Unfortunately, published information on the tumor and stromal composition of uveal melanoma metastases is limited. A recent article described the immune cell composition of 21 metastatic uveal melanomas, including both hepatic (n=17) and extra-hepatic (n=4) metastases, and correlated the outcome with patient response to various systemic and local treatments (immune checkpoint inhibition and/or chemoembolization with irinotecan charged microbeads), and survival. This led to the conclusion that the percentage of intratumoral granzyme B positive CD8 T cells (activated cytotoxic T lymphocytes) was a prognostic indicator. They also showed that the intra-tumoral density of CD163 positive tumor-associated macrophages (generally immunosuppressive M2-like) was higher in liver metastases when compared to extra-hepatic metastases[15]. Unfortunately, the number of extra-hepatic metastases was small (n=4) and there were no matched samples of both types of metastases from one patient. It would be interesting to validate these findings in a larger group of uveal melanoma patients, including multiple matched samples.

The most recent new treatment option for patients with irresectable uveal melanoma is systemic therapy with tebentafusp monotherapy. This immune-mobilizing monoclonal T cell receptor is a fusion of a soluble affinity-enhanced HLA-A*02:01-restricted T cell receptor for a glycoprotein 100 peptide (gp100) which is fused to an anti-CD3 single-chain variable fragment. The recently published open-label, phase 3 trial, included 378 patients with metastatic uveal melanoma. The overall survival at 1 year was 73% in the tebentafusp group versus 59% in the control group (hazard ratio for death 0.51, 95% confidence interval 0.37-0.71)[16]. This has led to the approval of this new treatment option by the FDA and EMA.

Another promising type of treatment involving T cell engagement, might be adoptive cell therapy. A first stage and ongoing expansion stage of a phase 2 trial with adoptive

cell therapy in uveal melanoma showed that seven of the 20 evaluable patients had an objective tumor regression (6 partial response, 1 complete response)[17]. There was a positive association between the frequency and absolute number of tumor-reactive tumor infiltrating lymphocytes (TIL) in the infusion product and response to treatment.

These specific TIL were determined by the sum of flow cytometric measurements of OX-40 positive CD4 T cells and CD137 positive CD8 T cells, following co-culture of the TIL with cryopreserved autologous tumor digests (when available).

Additionally, the absolute interferon-gamma production of these TIL following co-culture also seemed associated with response to treatment. No difference was observed in the number of non-synonymous mutations harboured by responding versus non-responding patients, in both groups the mutational burden was low[17]. Therefore, the question arises what is actually recognized by the tumor-reactive TIL. Are these, for example, neo-epitopes that have derived from the few somatic mutations present in the metastatic uveal melanoma? It will be interesting to further elucidate the specificity and identify the targets recognized by these reactive TIL in responding patients. At the same time it would be of importance to determine if TIL in non-responding patients are suppressed by the expression of immunomodulatory molecules that lead to T cell suppression, like Galectin-3, PD-L1, CTLA-4, Indoleamine 2.3-Dioxygenase-1, and Lymphocyte Activating 3, as was shown in a recent article in primary uveal melanoma[18].

Currently, two phase II trials are ongoing evaluating the efficacy of adoptive cell therapy in a larger cohort of amongst others uveal melanoma patients. The first trial will study 47 patients with metastatic uveal melanoma, who will be treated with a lymphocyte depleting preparative regimen followed by TIL and high-dose intravenous aldesleukin (NCT03467516). The second trial aims to determine whether the addition of dendritic cell vaccination to the combination of lymphodepleting chemotherapy, high-dose IL-2 and TIL leads to sustained persistence of the infused T cells when compared to lymphodepleting chemotherapy, high-dose IL-2 and TIL. This trial specifically includes patients with uveal melanoma, alongside patients with cutaneous melanoma (NCT00338377). In the first report on one of the different cohorts of the trial, the authors did not show a difference in the persistence of MART-1 TIL between the two groups. However, in the small group of 18 patients in total it seemed that there might be a better clinical response in the combination group (4/8 versus 3/10). Unfortunately, no uveal melanoma patients were included in this initial report[19]. The fact that more studies in metastatic melanoma include uveal melanoma in their inclusion criteria seems hopeful. This could potentially lead to new treatment combinations with adoptive cell therapy in this specific subgroup of melanoma patients.

Unfortunately, the reported durability of the clinical responses following TIL therapy in uveal melanoma is relatively short compared to the responses seen in cutaneous melanoma. A possible explanation for this might be that the infused T cells are suppressed by the intra-tumoral M2-like macrophages. In order to support these infused T cells, combining this treatment with M2 targeting therapy might be necessary to overcome the immune suppressive environment in hepatic metastases of uveal melanoma. Several treatment options have been described that can induce a M2 to M1-phenotype macrophage repolarization, including local low-dose irradiation[20] and tumor vaccines formulated with GM-CSF[21,22]. Recently, targeting of M2-like tumor-associated macrophages with a hybrid peptide MEL-dKLA was used *in vivo* in a lung cancer model[23], and in a breast cancer model where it enhanced the PD-L1 mediated anti-tumor effect[24]. Multiple clinical trials are currently ongoing with macrophage targeting agents. For melanoma patients these include trials with CD40 agonists and CSF-1 receptor inhibitors[25].

Interestingly, a recent abstract on the NCT03123783 clinical trial with a CD40 agonist showed that 6 out of 33 patients with anti-PD-1 refractory metastatic cutaneous melanoma developed a partial response to the combination of anti-PD-1 and a CD40 agonist[26]. The same was seen in a mouse model of another immunologically desert tumor; pancreatic carcinoma. The authors conclude that the CD40 agonist leads to priming of both CD4 and CD8 T cell subsets, while anti-PD-1/anti-CLTA-4 treatment removes negative feedback signals for these newly primed T cells[27]. Multiple trials are ongoing in both immunologically hot and cold tumors to further study the effect of CD40 agonists in combination with immune checkpoint inhibition.

Another potentially promising adoptive T cell therapy for uveal melanoma, might be with Chimeric Antigen Receptor (CAR)-T cells. Hereby, the T cell receptors (TCR) of isolated peripheral T cells are further engineered to express extracellular antigen recognition domains targeting a tumor-specific cell surface protein[28]. So far, treatment with CAR-T cells has shown great promise in hematologic malignancies, including acute lymphoblastic leukemia, chronic lymphocytic leukemia, lymphoma, and multiple myeloma. In cutaneous melanoma multiple potential stable target antigens for CAR-T cells have been identified, including CD20, disialoganglioside GD2, CD171[29], chondroitin sulfate proteoglycan 4 (CSPG4)[30], and HER2[31]. Currently, there are no clinical trials ongoing for CAR-T cell therapy in uveal melanoma. Based on data from The Cancer Genome Atlas, HER2 mRNA is expressed at appreciable levels by both cutaneous and uveal melanoma. In the pre-clinical trial where it was shown that CAR-T cells directed against HER2 could kill cutaneous melanoma cells *in vitro* and in a humanized mouse model, also two uveal melanoma cell lines were included. These commercially available cell lines were sensitive to HER2 CAR-T cells[31].

A challenge for CAR-T cell therapy in solid tumors versus hematological malignancies, is that the tumors are poorly infiltrated by immune cells, that tumor microenvironment blocks the effect, that the infused cells become exhausted before they can eradicate the tumor, or that the targeted antigen is not uniformly expressed on the tumor cell surfaces or different metastases. In order to overcome these hurdles, recently the combination of an RNA vaccine and CAR-T cells targeting the same target (tight junction protein claudin 6) was studied in mice. This trial showed an enhanced efficacy of the infused CAR-T cells when combined with an RNA vaccine, designed for body-wide delivery of the CAR antigen[32]. A recent study showed that also an intracellular oncogenic transcription factor (WT1) could be targeted by CAR-T cells[33]. Another treatment option might be to target the malignant melanoma stem cells. Markers for this specific subgroup of melanoma cells include the previously named CD20[34] and CD133[35]. At writing, a phase 1 trial is ongoing that studies the safety of CD-targeting CAR-T cells in advanced melanoma patients (NCT03893019). It will be interesting to see the future developments in the treatment for metastatic uveal melanoma. When compared to cutaneous melanoma, it seems that more hurdles have to be overcome to reach lasting clinical responses.

In this discussion several promising treatment options were already described, including treatment with the combination of PHP and immune checkpoint inhibitors. The first promising results from an initial clinical trial of autologous T cell transfer in uveal melanoma were also described. In order to reduce the T cell suppression by intra-tumoral M2-like macrophages adoptive cell therapy might be combined with MEL-dKLA. In order to enhance the release of cancer cell antigens due to cell death, to increase MHC class I expression, and to trigger more intratumoral antigen-specific T cells, the harvesting of TIL might be preceded by melphalan treatment[36]. For example by combining TIL treatment with PHP.

Meanwhile, the search for an suitable target for CAR-T cell therapy continues in uveal melanoma. As was described earlier, the HER2 directed CAR-T cells might hold great promise. We will have to await further trials to verify the effect of these cells in uveal melanoma. And following these reports, combinations with RNA vaccines targeting the same target might be considered.

Part II: From bench to registry and back

The current evidence pyramid visually depicts the evidential strength of different research types. At the foundation of the pyramid usually animal and laboratory studies are depicted. This is followed by case reports/series, case control studies, cohort studies, and at the top of the pyramid randomized controlled trials are placed. These

studies are the ones that can lead to market approval and the widespread use of the different interventions.

However, these large phase III randomized controlled trials do not typically represent the entire population of patients that will receive the medicinal product. A recent study comparing systemically treated patients with advanced melanoma showed that 40% of the patients treated in the Netherlands would not have been eligible for inclusion in phase III trials[37]. The inclusion and exclusion criteria for these trials exclude a vast number of patients, based on for example: age, disease progression, brain or leptomeningeal metastasis, comorbidity, and use of (immune-modulating) medication.

Medical registries were initially mainly used for calculating valuable epidemiological data, like incidence, prevalence, and mortality. However, these registries have evolved, and can now include data on adverse events, quality of life, laboratory values, and medical history of the patient. The Dutch Melanoma Treatment Registry (DMTR) is a national registry databases that includes information on all advanced melanoma patients in the Netherlands. In **Chapter 5**, **6**, and **7** I used the data from the DMTR to study the safety and efficacy of systemic treatments for advanced melanoma patients in different subgroups.

Stepping away from the widely used evidence pyramid that depicts animal and laboratory studies at the bottom, I would like to argue that real-world registry data could also be used to create new fundamental research questions. In **Chapter 6** of this thesis we showed that there were distinct differences in primary tumor characteristics, and tumor mutations between patients 15-39 years of age (AYA) and older adults. We showed that the common BRAF mutation was even more prevalent in the AYA age group. I hypothesize that this may implicate that the prevalence of mutations in more melanoma driver genes will differ between AYA and older patients. In order to compare these mutational profiles it would be interesting to have access to whole-genome sequencing data (especially single-nucleotide variants, multiple-nucleotide variants, small insertions and deletions, structural variants, UV radiation related mutation signatures, and the median tumor mutational burden). Currently, the treatment regimen is roughly the same for every metastatic melanoma patient, except for BRAF treatment that is dependent on the presence of the BRAF V600 mutation. Based on the findings presented in **Chapter 6**, I hypothesize that early onset melanoma is a separate entity with a different prevalence of mutations in melanoma driver genes, when compared to older patients. Studying these differences could help identify potential targetable genomic differences between young and older patients with metastatic melanoma, which in turn could lead to age-specific mutational analysis in the future.

In the current era of medicine we are fortunate to have databases that collect these type of whole-genome sequencing data. In the Netherlands, this data is collected by the Hartwig Medical Foundation. Currently, their data is being analyzed to correlate the findings in our nationwide registry with more in-dept sequencing data. The aim is to understand the exact differences and identifying the potentially targetable genomic differences between young and older patients with metastatic melanoma.

Investigating patient data on a national, or even international scale, will not only be beneficial for patients with cutaneous melanoma. Data-registries and collaborations will have an even greater benefit for patients with rare cancers. Approximately 200 malignancies are defined as rare cancers (6 or less cases per 100.000). In Europe, rare cancers account for 24% of all malignancies[38,39]. As Nathan et al. showed in their phase 3 trial with tebentafusp for patients with metastatic uveal melanoma, randomized studies are possible for rare cancer types albeit requiring large international consortia[16]. A potential way of reducing the number of patients with rare cancers that have to be included in these trials, is the use of "historical cohorts". In this thesis, we included nation-wide data on uveal melanoma that can be used as such (**Chapter 4**). I would encourage registries with rare cancer types to join forces on an international level. Combining survival data on such a large scale will make it possible to provide "historical cohorts" for researchers, leading to less patients being treated with "standard of care" therapies and possibly more trials for patients with rare malignancies.

Another benefit of joining forces on an international level could be to compare treatment strategies and stage-specific survival of patients with melanoma in, for example, Europe. Over the past decades treatment options have changed for patients with melanoma. However, not all countries in Europe added these treatments to their standard of care at the same point in time. It would therefore be interesting to see if survival changed since the introduction on these new treatment options. In addition "country" could be used as an instrumental variable in comparing neighboring countries to identify an association between treatment strategy and survival.

One of the key questions in medical oncology was whether patients with a preexisting autoimmune disease could be treated with ICI. Treating oncologists worldwide feared potential flares in patients with an already overactive immune system. Therefore, patients with this type of comorbidity were excluded from the phase III trials that led to market approval of both anti-CTLA-4 and anti-PD-1 treatment. However, using the DMTR database it was possible to publish data on this specific group of patients and to compare both treatment outcome and overall survival with a large group without an autoimmune disease (**Chapter 5**). Showing that ICI can be prescribed to patients with common autoimmune diseases of endocrine and rheumatologic origin, has had

a major clinical impact worldwide. This was evidenced by the interest for the subject on multiple (international) conferences and the reports from multiple clinicians that they indeed are now less hesitant to treat patients with common autoimmune diseases with anti-CLTA-4 or anti-PD-1.

Another important aspect for which these large registries can be used is validation of scoring systems or models that were based on (smaller) trials. One of the first examples is shown in **Chapter 7.2**. By using data from the DMTR, we found that a previously published prediction model for response to anti-PD-1 could not be validated. I can't emphasize enough how important these kinds of validation attempts are. Many researchers try to create an appealing and easy scoring system for response to drugs. However, as we have learned from amongst others the Cancer Immunity Cycle, tumor regression is (unfortunately) not so easily reached nor defined.

One of the variables used in the prediction model was gender. For many years it has been known that women have a survival advantage over men with melanoma. Many possible explanations have been studied, including; behavioral differences leading to earlier detection in women, possible differences in mitotic rate, and BRAF mutation rate. Interestingly, previous studies already showed that the survival advantage for women became smaller in patients with more advanced disease. A recently published theory states that women are prone to stronger immunoediting in early tumor development. This strong initial immune response leads to the fact that when tumors have grown and metastasized the effectively-presented driver mutations are already significantly depleted. This renders advanced melanomas in women less visible to the immune system and therefore more difficult to treat with ICI[40]. In line with this hypothesis, it was found that in (mostly) metastatic melanoma patients the tumor mutational burden was lower in women when compared to men[41-43]. Using gene expression pathway analysis, a recent report on mostly stage IIIB and IIIC melanoma patients showed that tumors from women were enriched in immune related pathways when compared to tumors from men. Apart from CD8 and CD4 T cell pathways, this also included the regulatory T cell pathway. However, when peripheral blood was analysed, it was shown that women had a higher percentage of CD3 positive cells, while men had higher percentages of monocytes and trends towards higher percentages of regulatory T cells[44].

This could be a possible explanation for some findings presented in **Chapter 7.1**. The reported overall survival advantage of 10% for women when compared to men, was no longer present when only patients treated with ICI for advanced melanoma were analyzed. The primary melanomas of women were thinner when compared to men, and female patients had a longer time gap between primary disease and the development of advanced disease. Is this longer time gap explained by the fact that the

primary tumors were earlier detected, and therefore thinner, in women. Or does early strong immuno-editing play a role? If the theory about early immunoediting is true, we would expect to see a difference in response between men and women when ICI are given at an earlier stage.

Recently, the Checkmate-238 and EORTC 1325/Keynote-054 trials led to registration and approval of anti-PD-1 as adjuvant systemic treatment in resected stage III and IV melanoma. Interestingly, in 2021 De Meza et al. published the first data on adjuvant anti-PD-1 treatment in patients with melanoma using data from the DMTR. In their univariate Cox regression model women had a better recurrence-free survival (HR 0.64, 95% CI 0.48-0.87). Factors that were associated with recurrence-free survival in univariate Cox were included; sex, tumor stage, ulceration present in primary melanoma, Breslow thickness, and BRAF-V600 mutation status. These factors were included in a multivariate Cox in the supplemental material. Women recurrence-free survival advantage remained (HR 0.69, 95% CI 0.48-0.97)[45]. A comparable result was seen in the earlier mentioned trial that showed that women with a stage IIIB an IIIC had a higher infiltration with immune cells compared to men. When these women were treated with adjuvant anti-CTLA-4 they showed both a longer overall survival and relapse free survival[44]. Although these data cannot directly be compared with our data in **Chapter 7**, as age and patient performance score were not included, these results strengthen the theory that women might benefit more from early treatment with ICI, possibly due to the strong immune response early in disease development.

Neoadjuvant treatment in melanoma is not (yet) a registered treatment for melanoma. Therefore, we turn to the data from the recently published phase II OpACIN-neo and OpACIN neoadjuvant ICI trials[46,47]. The currently published data from these trials mainly focusses on the pathologic response rate following three different ICI treatment regimens. In the percentage of pathologic responses the OpACIN-neo did not show a significant response difference in response rate between women (62%; 95% confidence interval 45-78) and men (84%; 95% confidence interval 70-93)[47]. In coming years it would be very interesting to analyze the neoadjuvant data on a national scale, in order to really make a head to head comparison in the survival advantage of women versus men following neoadjuvant, adjuvant and regular ICI treatment.

Part III: Time for TIL

In this thesis I presented the data from our phase I/II clinical trial with adoptive T cell transfer in combination with low dose interferon-alpha (**Chapter 8**). It was shown that this combination was safe and could lead to clinical results, even in patients who already had progression of their melanoma under immuno- and targeted therapy. Interestingly, we found that a large portion of infused T cells expressed PD-1 on their surface[48].

These findings formed the basis for our currently ongoing trial, where we combine anti-PD-1, interferon-alpha and adoptive T cell transfer (**Chapter 9**)[49]. In this thesis the first preliminary data is published on both safety and efficacy of this novel treatment combination. We conclude that this combination can safely be prescribed to patients with melanoma who have already progressed on all standard of care treatment options. Additionally, several heavily pre-treated patients still show a clinical response.

Currently, the first phase III trial comparing TIL with ipilimumab has completed inclusion. The preliminary results show that the progression free survival of patients receiving TIL was significantly longer when compared to patients who were treated with ipilimumab. This could pave the way for TIL treatment to become part of the standard of care treatment options for patients with melanoma.

A possible way to further improve the clinical outcome of adoptive T cell therapy lies in the selection of the metastatic site to culture these cells from. Currently, this selection process is solely made on the basis of which metastases has the best access for surgical removal. However, we know that the presence of large numbers of infiltrating lymphocytes in the primary tumor, metastatic lesion, stroma, and (draining) lymph node has been shown to hold predictive value with respect to the natural history of melanoma[50-56]. It was already shown that the presence of higher concentrations of CD8+ lymphocytes in the (single) tumor from which TIL for adoptive T cell therapy were harvested, was correlated with a better survival[14]. As TIL play a central role in the response, an effective method to select patients and predict responses is crucial. Therefore, over the past years multiple mouse-studies and the first phase-I (human) clinical studies have been published using immune-PET/CT with zirconium-89 (^{89}Zr) labeled CD8+ antibodies to quantify tumor infiltration *in vivo*. This has the advantage that the technique is non-invasive and does not suffer from sampling error due to heterogeneity: the whole tumor burden can be quantitatively assessed. A recent study showed that a ^{89}Zr-labeled human CD8-specific minibody could detect CD8+ lymphocyte infiltration by small animal immuno-PET imaging in a xenograft mouse model[57]. It was shown that the radiopharmaceutical distribution not only spatially matched immunohistochemistry for CD8+, but also quantitatively. The first in human imaging study with this anti-CD8 minibody showed the procedure to be safe and confirmed a correlation between high radiopharmaceutical uptake determined by immuno-PET/CT and CD8 staining using immunohistochemistry[58]. In order to take adoptive T cell therapy a step further, I believe it would be promising to use radiolabeled CD8 antibodies as a selection tool for the lesion to culture T cells from to be used in adoptive cell therapy.

In order to further improve the effect of TIL therapy, it would also be beneficial to select TIL that respond to neo-antigens[48,59]. Detection of these neo-antigens can

be performed using genome and RNA sequencing data from the treated patients in comparison to healthy tissue. Using algorithms for amongst others HLA-binding, stability, and epitope foreignness the most potent neo-epitopes can be selected. Selecting and expanding only those TIL that respond to these neo-epitopes would yield better clinical results[60].

As the process of neo-epitope selection is both time-consuming and costly, one would ideally select TIL based on (a combination of) activation-induced surface markers. Over the years many surface markers have been studied[61]. CD137 is upregulated on CD8 and CD4 T cells following antigen-specific stimulation[62,63]. It was shown that the expanded CD137 positive fraction of TIL had been enriched for neoantigen-specific T cells[64]. Other markers that were suggested and exhibited antitumor activity were; PD-1, CD39, and CD103. Particularly, the combination of the latter two was shown to identify tumor-reactive CD8 T cells[65]. A recent comparative study on surface markers in human high-grade serous ovarian tumor samples showed that the antitumor abilities of PD-1, CD103 and CD39 positive T cells was mainly derived from a subset of CD137 expressing TIL[66].

Currently, there is a trial ongoing in the Erasmus Medical Center studying adoptive T cell therapy with autologous T cells, gene-engineered to express the MAGE-C2 antigen (NCT04729543). This is a tumor specific target in 40% of melanomas and 20% of head and neck squamous cell carcinomas[67-69]. As MAGE-C2 is not expressed in healthy tissues, except for the gonads, it will be interesting to see whether this treatment protocol indeed shows less toxicity when compared to previous trials with differentiation antigens, including MART-1, gp100, CAE and p53[70-72].

The past decade in medicine belonged to ICI with anti-CTLA-4 and anti-PD-1. Their development and clinical implementation has made a great impact on our understanding of cancer pathogenesis, and has importantly improved survival of patients with many different tumor types. However, we are now at the beginning of a new era, where we will face the challenges of immunotherapy-resistance.

Discussed here were some promising new developments for patients with uveal and cutaneous melanoma. Where cutaneous melanoma treatment will mostly have to battle secondary immunotherapy resistance, uveal melanoma treatments will have to overcome primary immunotherapy resistance. In order to offer TIL therapy to both groups of patients, immunologists, oncologists, pathologists, pharmacists, radiologists, and epidemiologists will have to join forces to determine the best treatment add-on to TIL therapy for these two very different types of melanoma.

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Appendix

Nederlandse samenvatting

Net als met alles in het leven, draait het ook in de oncologie om balans. Het lichaam heeft het eigen immuunsysteem dat het hoort te beschermen tegen ziekteverwekkers en kanker. Bij kanker wordt het immuunsysteem vaak geremd, waardoor de kankercellen kunnen ontsnappen en groeien. Door het immuunsysteem te stimuleren, kunnen kankercellen worden opgeruimd. Bij een overactief immuunsysteem ontstaan er echter auto-immuunziektes, waarbij het immuunsysteem de eigen gezonde cellen aanvalt.

Door opgelopen schade (bijvoorbeeld door de zon) kunnen bepaalde huidcellen (melanocyten) ontsporen. Dit komt door genetische defecten die ontstaan in deze huidcellen. Ze gaan dan ongeremd delen en zorgen ervoor dat ze uiteindelijk kunnen gaan uitzaaien. Deze kwaadaardige vorm van huidkanker wordt melanoom genoemd.

De genetische defecten in de huidcellen hebben in de behandeling ook een voordeel, ze zorgen ervoor dat deze cellen door het immuunsysteem van de patiënt kunnen worden opgepikt. Hoe meer genetische defecten er zijn binnen een cel, hoe lichaamsvreemder deze wordt.

Immuuntherapie heeft als doel het eigen immuunsysteem de tumor te laten aanvallen en op te ruimen. Dat kan op veel verschillende manieren. Veruit de bekendste is het weghalen van een rem op het immuunsysteem (immuun checkpoint inhibitoren). Doordat het eigen immuunsysteem van de patiënt minder geremd wordt, kunnen de afweercellen de tumor beter aanvallen.

In **hoofdstuk 2** van dit proefschrift heb ik beschreven hoezeer oog- en huidmelanoom van elkaar verschillen, terwijl ze uit dezelfde type cel ontstaan; de melanocyt. Zo laten de tumoren een heel ander patroon van genetische defecten zien. Naast een ander patroon wordt ook gezien dat oogmelanoom minder genetische defecten heeft en dus mogelijk minder lichaamsvreemd is.

In **hoofdstuk 3.1** en **3.2** gaan we verder in op dat laatste punt. Als er weinig defecten zijn, zijn er dan nog wel genoeg aangrijpingspunten voor het immuunsysteem om het oogmelanoom te herkennen en aan te vallen? Oogmelanoom is een zeldzame vorm van kanker. Daarom hebben we in de studies beschreven in deze hoofdstukken de krachten gebundeld met andere ziekenhuizen in Nederland. Samen laten we zien dat immuun checkpoint inhibitoren, anti-CTLA-4 en anti-PD-1, niet goed werken in patiënten met oogmelanoom. Een belangrijke uitkomst, omdat deze behandelingen voor veel bijwerkingen kunnen zorgen.

Hoe patiënten met een vergevorderd oogmelanoom dan wel worden behandeld, wordt beschreven in **hoofdstuk 4**. Hier wordt wederom gebruik gemaakt van Nederlandse patiëntgegevens, ditmaal van de Dutch Melanoma Treatment Registry (DMTR). Deze organisatie registreert de gegevens van alle patiënten in Nederland met een vergevorderd melanoom. Daarbij worden de gegevens ingevoerd door getrainde datamanagers, en nagekeken door oncologen uit de 14 behandelcentra in Nederland. Gegevens van de tumor, de behandeling en het effect worden bijgehouden. Door deze gegevens te analyseren laten we zien dat het hebben van uitzaaiingen in de lever samenhangt met een slechtere overleving vergeleken met het hebben van een uitzaaiing op een andere plek in het lichaam. Tevens laten we zien welke behandelingen patiënten krijgen en hoe de overleving van deze patiënten is.

Een belangrijk verschil tussen dit artikel en veel andere wetenschappelijke studies, is dat een landelijke registratie alleen informatie geeft over welke keuzes er gemaakt zijn en niet waarom. De keuze om wel of niet te behandelen wordt gemaakt op basis van veel gegevens. Soms zijn die heel tastbaar, soms zijn ze achteraf moeilijk te bepalen. Biologische leeftijd is een voorbeeld: een oudere en verzwakte patiënt zal samen met de arts zelden kiezen voor de zwaarste behandeling met de meeste bijwerkingen. Het lastige is vervolgens dat een registratie niet iemand zijn biologische leeftijd vermeldt, maar alleen de kalenderleeftijd van de patiënt.

Toch blijft het heel belangrijk om gegevens uit registratiedata te publiceren. Het is namelijk een belangrijke manier om betrouwbare informatie te krijgen over hoe de overleving en bijwerkingen van bepaalde (nieuwe) medicijnen zijn bij patiënten buiten de strikt gereguleerde studies.

De grote studies waar zowel belangrijke wetenschappelijke tijdschriften als landelijke media veel aandacht aan besteden, zijn zogeheten gerandomiseerde studies. Deze studies hebben strikte richtlijnen voor wie er “geschikt” is om deel te nemen. Vaak wordt er geselecteerd op niet al te oude patiënten, die weinig verschillende medicijnen gebruiken en niet al te veel andere ziektes hebben. Binnen deze groep wordt er vervolgens meestal via loting bepaald welke behandeling een patiënt ontvangt.

Deze benadering heeft veel voordelen. Je hoopt namelijk de effectiviteit en veiligheid van een nieuw middel zo betrouwbaar mogelijk te onderzoeken in de door jou geselecteerde groep patiënten. Maar in hoeverre gelden de gevonden resultaten ook voor patiënten die niet binnen deze strikte kaders vallen? Deze vragen worden behandeld in deel twee van dit proefschrift.

In **hoofdstuk 5** gebruik ik registratiedata uit de DMTR om te bepalen in hoeverre behandeling met een immuun checkpoint inhibitor veilig gegeven kan worden

aan patiënten met een auto-immuunziekte. Uit angst voor het verergeren van de al bestaande auto-immuunziekte mocht deze groep patiënten niet meedoen aan grote gerandomiseerde studies met immuun checkpoint inhibitoren. Derhalve wisten oncologen wereldwijd niet of dit middel veilig was voor deze groep. Daarom kreeg deze groep patiënten soms wel, en soms geen immuun checkpoint inhibitor behandeling.

Verder wordt getoond dat patiënten met vaak voorkomende auto-immuunziektes op reumatologisch en endocrinologisch (hormoonaandoeningen) vlak veilig kunnen worden behandeld met de eerdergenoemde immuun checkpoint inhibitoren. Bij patiënten met inflammatoire darmziekte (IBD) is extra voorzichtigheid wel geboden. In deze groep werd gezien dat patiënten vaker last kregen van een darmontsteking, of vervroegd moesten stoppen met de medicatie.

Een andere groep die relatief ondervertegenwoordigd is in de "reguliere" studies zijn ouderen en jongeren. Daarom staat **hoofdstuk 6** in het teken van de jongere patiënt met een vergevorderd melanoom. Het onderzoek laat zien dat de genetische defecten die we zien bij melanomen deels leeftijdsafhankelijk zijn. Een goed voorbeeld hiervan is de BRAF-mutatie. Deze leidt tot ongeremde celdeling en wordt met name bij jongere patiënten met een melanoom vaak gezien. Sommige medicijnen tegen melanoom grijpen aan op deze specifieke mutatie, dit wordt doelgerichte therapie genoemd. Doordat er een blokkade wordt gevormd, wordt ook de ongeremde celdeling gestopt.

Zodra er met deze doelgerichte therapie of met immuun checkpoint inhibitoren wordt gestart zien we weinig verschil meer tussen de oudere en de jongere patiënt qua bijwerkingen en ziekte-specifieke overleving. Het is belangrijk om naar dit type overleving te kijken, aangezien over het algemeen jonge mensen nog langer te leven hebben dan oudere mensen. Door naar ziekte-specifieke overleving te kijken corrigeer je voor dit gegeven.

Van oudsher leven vrouwen langer dan mannen, al is dat verschil de afgelopen jaren steeds kleiner geworden. In **hoofdstuk 7.1** is te lezen dat vrouwen met een vergevorderd melanoom ook langer leven. Deze overlevingswinst wordt met name gezien bij de groep patiënten met de eerdergenoemde BRAF-mutatie. Overigens zijn vrouwen in het algemeen ook jonger als er vergevorderd melanoom wordt geconstateerd. Bij behandeling met immuun checkpoint inhibitoren wordt er geen verschil gezien in overleving tussen mannen en vrouwen.

Dat laatste gegeven sprak een eerder gepubliceerd model tegen, waarbij werd gedacht dat onder andere aan de hand van geslacht kon worden voorspeld hoe goed het resultaat van de immuun checkpoint inhibitor behandeling zou zijn. In **hoofdstuk 7.2** hebben we dit model geprobeerd te staven aan de gegevens uit de DMTR. Hieruit bleek

dat de realiteit zich niet laat vangen in een 0 tot 7 puntensysteem, enkel gebaseerd op klinische gegevens van de patiënt.

In de geneeskunde houden we van dit soort "makkelijke" scoring systemen, omdat ze je handvatten bieden voor het voorlichten van de patiënt over zijn kansen. Daarnaast wil je een patiënt een behandeling besparen als het hem of haar geen baat zal brengen, maar wel bijwerkingen. Dit artikel laat zien dat registratiedata ook heel belangrijk zijn om dit soort scoring systemen te checken. Ze worden namelijk vaak gemaakt op basis van studie data met wederom geselecteerde patiënten, maar zoals al eerder beschreven valt een groot deel van de patiënten in de spreekkamer niet binnen die strenge selectiecriteria.

In het derde deel van dit proefschrift gaat het voornamelijk over een specifieke vorm van celtherapie voor patiënten met gemetastaseerd melanoom. Bij adoptieve celtherapie worden afweercellen van de patiënt buiten het lichaam gekweekt en vermenigvuldigd. Door deze cellen buiten het lichaam te kweken, zich te laten vermenigvuldigen en vervolgens terug te geven aan de patiënt probeer je het eigen immuunsysteem nog meer strijders te geven om de tumor te bevechten. Deze behandeling is niet nieuw en wordt al sinds 1980 in studieverband gegeven. Van de tot nu toe gepubliceerde studies gebruiken veruit de meeste een voorbehandeling om het eigen immuunsysteem te onderdrukken, voordat de gekweekte extra eigen immuuncellen via het infuus worden toegediend. Zo zou er "ruimte" gecreëerd worden voor de opgekweekte cellen. Deze voorbehandeling gaat met behoorlijk veel bijwerkingen gepaard en zorgt er ook voor dat patiënten vaak enkele weken in het ziekenhuis opgenomen moeten worden.

In het LUMC gebruiken we al jaren een lichtere vorm van voorbehandeling, die patiënten thuis kunnen toedienen. Daardoor is ons behandelschema minder belastend, en is de opnameduur korter. **Hoofdstuk 8** laat zien dat de combinatie van adoptieve celtherapie met de lichte voorbehandeling middels het medicijn interferon-alfa goed verdragen wordt en leidt tot stabilisatie, verkleining of verdwijnen van de tumor in 10 van de 34 patiënten (29%). Belangrijk om te realiseren bij deze gegevens is dat de overgrote meerderheid van de patiënten al progressieve ziekte had op eerdere behandelingen tegen het gemetastaseerd melanoom. Dat betekent dat de tumor is blijven groeien tijdens en na eerdere behandeling met een immuun checkpoint inhibitor of doelgerichte therapie. De toegediende immuuncellen van patiënten die goed reageerden op de behandeling waren specifieker dan de toegediende immuuncellen van de patiënten die niet goed reageerden op de behandeling. Dat houdt in dat deze cellen alleen iets herkennen wat specifiek op de tumorcel van hun patiënt voorkomt, en wat niet op de tumorcellen van andere patiënten te zien is. Daarnaast viel in de studie op dat veel van de gekweekte immuuncellen een bepaalde marker op de oppervlakte van hun cel hadden. Een marker is een soort vlag

op het oppervlak van een cel. Cellen hebben meerdere markers op hun oppervlak, de combinatie van deze markers vertelt je wat voor soort cel het is, welke functionaliteit hij heeft, maar kan er ook voor zorgen dat de cel door bepaalde stoffen of cellen geremd kan worden. Bij de door ons opgekweekte cellen kwam de PD-1 marker vaak tot uiting op het oppervlak. Deze PD-1 marker is een immuun checkpoint, die ervoor kan zorgen dat de immuuncellen na toediening geremd worden. Hierdoor kunnen ze de tumor minder goed aanvallen.

Voor een succesvolle behandeling wil je niet dat de immuuncellen die je gekweekt hebt worden afgeremd door de tumor zodra je ze teruggeeft aan het lichaam. Daarom zijn de gegevens uit hoofdstuk 8 gebruikt om een nieuwe studie op te zetten. In **hoofdstuk 9.1** wordt de ACTME-studie beschreven. Deze studie is bedacht, geschreven en opgezet gedurende dit promotie traject. Patiënten met gemetastaseerd melanoom ontvangen hierbij de eerdergenoemde interferon-alfa voorbehandeling, gevolgd door gekweekte eigen immuuncellen, en daarnaast ook immuun checkpoint inhibitor anti-PD-1 om de remmende verbinding tegen te gaan die de tumor zou kunnen gebruiken. Het doel van deze studie is om de momenteel beschikbare immuuntherapieën op vier punten te verbeteren: 1) het aanleveren van meer immuuncellen die de tumor kunnen aanvallen, 2) het voorkomen dat de tumor aan immuuncellen kan ontkomen door de rem via PD-1 op te heffen, 3) het verminderen van de toxiciteit van adoptieve celtherapie door onder andere de lichtere voorbehandeling, en 4) het reduceren van de belasting voor patiënten doordat zij met de lichtere voorbehandeling niet meer opgenomen moeten worden en op de dagbehandeling hun infuus krijgen.

Allereerst zal de combinatie van immuuncellen met de immuun checkpoint inhibitor anti-PD-1 aan patiënten worden gegeven. Als die veilig blijkt, kan er door worden gegaan met de combinatie van de drie middelen. In totaal zullen 25 patiënten met de combinatie van de drie middelen worden behandeld. Op basis van het aantal patiënten waarvan de tumor langdurig stopt met groeien, of waarvan de tumor in formaat afneemt, zal worden besloten of deze studie naast veilig ook effectief is.

In **hoofdstuk 9.2** staan de eerste, voorlopige, gegevens van de ACTME-studie. De studie is succesvol door het eerste deel gekomen, waarbij we hebben laten zien dat de combinatie van de immuun checkpoint inhibitor anti-PD-1 met gekweekte immuuncellen veilig is. Ook de combinatie van anti-PD-1, gekweekte immuuncellen en interferon-alfa voorbehandeling blijkt veilig. Dit kalenderjaar zullen alle patiënten binnen de ACTME-studie behandeld zijn, waarna hopelijk over 1-2 jaar alle resultaten worden gepubliceerd.

We zien tot nu toe dat bij meerdere patiënten de tumor stopt met groeien of kleiner wordt onder behandeling. Daarnaast valt op dat bij sommige patiënten bepaalde

uitzaaiingen kleiner worden, terwijl andere groeien. Op basis van deze gegevens is het interessant om in de toekomst te kijken hoe de verschillende uitzaaiingen binnen 1 patiënt van elkaar verschillen en welke uitzaaiing het best gebruikt kan worden om immuuncellen uit te kweken.

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Curriculum Vitae

Monique Krystyna van der Kooij werd geboren in 1992 te Leiden. In 2010 behaalde ze haar gymnasiumdiploma aan het Mendelcollege in Haarlem. Ze volgde daarbij twee profielen – Natuur & Gezondheid en Natuur & Techniek, met geschiedenis en aardrijkskunde als extra vakken. Daarnaast was ze lid van de medezeggenschapsraad en de leerlingenvereniging, ook richtte ze de leerlingenraad op.

In 2010 keerde ze terug naar haar geboorteplaats om daar te starten met de studie Geneeskunde. Naast de reguliere vakken van de bachelor nam zij deel aan Honours College, waar zij extra cursussen bij de afdeling Klinische Epidemiologie combineerde met onderzoek op de afdelingen Tumor Genetica en Medische Oncologie. Op de afdeling Medische Oncologie leerde ze dr. H.W. Kapiteijn kennen, met wie ze sindsdien veel onderzoeken heeft opgezet.

Tijdens haar master werd Monique geselecteerd voor deelname aan het Leiden Leadership Programme van de Universiteit Leiden. In 2014 ging zij als student-minister van onderwijs naar de G20 voor studenten in Garmisch-Partenkirchen. Naast deze extra curriculaire activiteiten rondde Monique in 2016 haar studie Geneeskunde *cum laude* af.

Aansluitend hieraan begon ze met haar promotieonderzoek op de afdeling Medische Oncologie van het Leids Universitair Medisch Centrum (promotor: prof. S.H. van der Burg, co-promotoren dr. H.W. Kapiteijn en dr. ir. E.M.E. Verdegaal). Tijdens haar promotieonderzoek bleef Monique de samenwerking opzoeken met andere afdelingen, waaronder Klinische Epidemiologie, Tumor Immunologie, Pathologie, Nucleaire Geneeskunde en Medische Statistiek. Voor haar onderzoek schreef ze meerdere gehonoreerde subsidieaanvragen.

Na vier jaar fulltime als promovendus te hebben gewerkt, begon Monique in 2020 als arts niet in opleiding op de afdeling Interne Geneeskunde in het HagaZiekenhuis in Den Haag. Sinds 2022 volgt zij de opleiding tot internist aan het Leids Universitair Medisch Centrum. Ook is ze betrokken bij het schrijven van het vervolg van de klinische ACTME-studie die ze tijdens haar promotietraject heeft opgezet.

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De afgelopen 10 jaar heb ik mogen meemaken hoe de overleving van patiënten met gemetastaseerd melanoom sterk is verbeterd. Het was een eer en een groot plezier om hier mijn steentje aan te mogen bijdragen. Ik heb op veel verschillende afdelingen gewerkt, om zo dit diverse, grensoverschrijdende proefschrift te kunnen volbrengen. En daar ben ik meerdere mensen ontzettend dankbaar voor.

Sjoerd, vanaf het moment dat ik eind 2015 op mijn eerste gesprek bij jou kwam, en je me vroeg "Waar wil jij je PhD over doen?", tot op de dag van vandaag heb ik altijd met je kunnen sparren. Ik hoop dat we de komende jaren nog veel samen kunnen blijven werken en dat ik ook dan altijd op je eerlijke mening mag rekenen.

Ellen, toen ik met jou meeliep op de polikliniek in 2012 wist ik het zeker: melanoom onderzoek en internist-oncoloog worden, dat is de gouden combinatie. We hebben samen veelbelovende projecten opgezet en het mooiste is misschien nog wel dat het verhaal hier niet eindigt.

Els, samen met Marten, Lien en natuurlijk Linda hebben jullie me de fijne kneepjes op het laboratorium eigen laten maken. We kunnen trots zijn op alle T cel producten die we hebben gemaakt, de tumorcellijnen die gekweekt zijn en ik kijk uit naar de resultaten die uit onze klinische studies gaan komen.

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Lieve meiden van de K1-19 (GBG's): Astrid, Florine, Maxime, Rieneke en Stefanie, we zijn samen het hele LUMC doorgetrokken. Steeds maar weer verhuizend, eindigden we uiteindelijk in het Poortgebouw. Samen konden we alles aan, want we wisten: deze promotiedag komt er. Voor sommigen is hij al geweest, voor anderen volgt hij op korte termijn.

Inge, door jouw warme persoonlijkheid voelde ik me in 2012 direct thuis bij de Medische Oncologie. Dank je wel voor je luisterend oor en alle goede adviezen.

Mare, onze geschiedenis begon in 2016 tijdens mijn semi-artsstage op de Medische Oncologie. Waar een goede kop cappuccino wel niet toe kan leiden.

Guillaume, mijn "buurman". Op de eerste spelletjesavond met de collega's van het laboratorium klikte het meteen. Samen joegen we digitaal op celpopulaties en filosofeerden we over de wildste onderzoeksideeën.

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Het Dutch Melanoma Treatment Registry team voor het opzetten en bijhouden van deze waardevolle landelijke registratie. En de betrokken oncologen voor alle feedback op en discussies over de artikelen.

Het melanoom-team. Daar horen veel van de bovenstaande personen uit het laboratorium en de kliniek bij, maar daarnaast natuurlijk ook chirurgen, radiotherapeuten, nucleair geneeskundigen, radiologen, pathologen en verpleegkundigen.

De patiënten die deelnemen aan de klinische studies. Het was een eer om jullie T cellen te bereiden, en vaak zelf langs te brengen op de afdeling. Die kleine praatjes, de mooie woorden, en soms het moeten uitleggen dat een arts-onderzoeker geen artsen onderzoekt. Het maakte mijn dag en zorgde ervoor dat ik nooit het doel uit het oog verloor: jullie.

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