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Experimental treatment of patients with disseminated malignant melanoma

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Abstract

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Malignant melanoma (MM) is the deadliest skin cancer with an ever-increasing incidence. New treatments have improved the prognosis for patients with advanced MM. Still, most patients do not respond, and the side effects can be severe, underlining the need for better therapies.

The overall aim of this thesis was to evaluate new means to improve the treatment for patients with advanced MM. Immunostimulatory gene therapy (AdCD40L) was evaluated in a clinical study and BRAF-inhibitory treatment in rare cases of BRAF-mutated MM.

Due to its immunogenicity, MM is an attractive target for immunostimulatory gene therapy. AdCD40L is an adenovirus carrying the human gene for CD40 ligand, which in different ways can stimulate the immune system to combat cancer. We conducted a Phase I/IIa study with AdCD40L in patients with metastatic MM having received established treatments. In cohort 1 (n=6), four weekly, intratumoural AdCD40L injections were given. In cohort 2 (n=9), low dose cyclophosphamide was added to increase the immune response. Since irradiation may act synergistically with immunotherapy, patients in cohort 3 (n=9) also received a single fraction of radiotherapy (8 Gy). This fraction was given towards the lesion selected for injections.

The primary objectives were to assess the feasibility and safety of AdCD40L-treatment and secondarily its anti-tumour effects. Patients were thoroughly assessed for toxicity. The anti-tumour response was evaluated by imaging techniques (FDG-PET/CT, DW-MRI scans), tumour biopsies and blood tests. Plasma protein markers were measured with a multiplex platform. Another objective was to evaluate the potential of DW-MRI and FDG-PET/CT for prediction of AdCD40L treatment response, in terms of overall survival (OS).

AdCD40L was well tolerated with mild transient reactions. Local and distant responses in PET/CT scans along with a significantly better 6-month survival in the cohorts that received cyclophosphamide conditioning were observed. Effector lymphocyte responses were elicited. All patients had an increased T effector/T regulatory-cell ratio and death receptors were significantly up-regulated post therapy. Inflammatory cytokines and other plasma proteins were altered in favourable ways by the AdCD40L treatment. The analyses support that the functional DWI parameters may be better early predictors of OS than the established metabolic and morphologic criteria of FDG-PET/CT and CT/MRI, respectively.

In conclusion, the stimulation of the CD40 pathway to initiate anti-tumour immunity is a promising treatment alternative for MM patients. However, further studies with developed treatment schemes are warranted.

In the first report ever on treatment of a pregnant patient with a BRAF-inhibitor, the therapy was initiated in the second trimester. The treatment with vemurafenib enabled prolonged gestation, hence reducing the risk of immaturity-related complications. Further, we report the first case worldwide of a patient with metastatic conjunctival melanoma who benefitted from treatment with vemurafenib. Additional studies are needed to assess the efficacy of BRAF -inhibitors in the different subtypes of ocular melanoma.

Keywords: Malignant melanoma, AdCD40L, immunotherapy, proteomics, DW-MRI, FDG-PET/CT, prediction, early response, BRAF-inhibitor, vemurafenib

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“The natural healing force within each of us is the greatest force in getting well.”

Hippocrates

«Οι φυσικές δυνάμεις που έχουμε μέσα μας, είναι οι πραγματικοί θεραπευτές της νόσου.»

*To Sofia and Alexander.
Such a perfect little arrangement of atoms.*

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Maleka, A.*, Loskog, A.*, Mangsbo, S., Svensson, E., Lundberg, C., Nilsson, A., Krause, J., Agnarsdóttir, M., Sundin, A., Ahlström, H., Tötterman, T.H.**, Ullenhag, G.J.** (2016) Immunostimulatory AdCD40L gene therapy combined with low-dose cyclophosphamide in metastatic melanoma patients. *British Journal of Cancer*, 2016 Apr 12;114(8):872-80 (*shared first position, **shared senior position)
- II Schiza, A., Wenthe, J., Mangsbo, S., Eriksson, E., Nilsson, A., Tötterman, T.H., Loskog, A., Ullenhag, G.J. (2017) Adenovirus-mediated CD40L gene transfer increases T effector/Tregulatory cell ratio and up-regulates death receptors in metastatic melanoma patients. *Journal of Translational Medicine*, 2017 Apr 20;15(1):79
- III Schiza, A., Irenaeus, S., Ortiz-Nieto, F., Loskog, A., Tötterman, T.H., Sundin, A., Ullenhag, G.J.*, Ahlström, H*. Diffusion-Weighted MRI May be Better than FDG-PET/CT to Assess Immunotherapy Response in Patients with Metastatic Melanoma. Manuscript (*shared senior position)
- IV Maleka, A., Enblad, G., Sjörs, G., Lindqvist, A., Ullenhag, G.J. (2013) Treatment of Metastatic Malignant Melanoma With Vemurafenib During Pregnancy. *Journal of Clinical Oncology*, 2013 Apr 10;31(11):e192-3
- V Maleka, A., Åström, G., Byström, P., Ullenhag, G.J. (2016) A case report of a patient with metastatic ocular melanoma that was successfully treated with the BRAF inhibitor vemurafenib. *BMC cancer*, 2016 Aug 12;16:634

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Contents

Introduction	9
Malignant melanoma	9
Background	9
Epidemiology	9
Pathogenesis	10
Types of melanoma	11
Staging and risk assessment of cutaneous malignant melanoma	12
Treatment of cutaneous malignant melanoma	15
Treatment of metastatic ocular melanoma	24
Radiological response evaluation	29
Tumour immunology	30
Tumour microenvironment	31
Immunosurveillance	32
The “cancer immunity cycle”	32
Immunoediting and immune inhibitory mechanisms	35
CD40 ligand and AdCD40L	39
Conditioning	42
Aims of the Doctoral Project	44
Subjects and Methods	45
AdCD40L trial	45
Study scheme	45
AdCD40L medicinal product	45
Paper I and II	46
Immunological analyses	46
Statistical analyses	47
Paper III	47
Radiological parameters	47
Statistical analyses	48
Vemurafenib case reports	48
Paper IV	48
Paper V	48

Results and Discussion.....	49
Paper I	49
Paper II	51
Paper III	52
Paper IV	56
Paper V	57
Concluding Remarks	58
Future Perspectives	59
Sammanfattning på svenska	60
Περίληψη στα Ελληνικά	63
Acknowledgments.....	66
References	68

Abbreviations

ACT	Adoptive Cell Transfer
ADC	Apparent Diffusion Coefficient
AJCC	American Joint Committee on Cancer
APC	Antigen Presenting Cell
CD	Cluster of Differentiation
CM	Conjunctival Melanoma
CT	Computed Tomography
CTL	Cytotoxic T-Lymphocyte
CTLA	Cytotoxic T-Lymphocyte-Associated Protein
D	True Diffusion Coefficient
D*	Perfusion-Related Coefficient
DCs	Dendritic Cells
DW-MRI	Diffusion-Weighted - Magnetic Resonance Imaging
ERK/MAPK	Extracellular signal-Regulated Kinase/Mitogen Activated Protein Kinase
f	Perfusion Fraction
FDG-PET/CT	¹⁸ F-Fluorodeoxy-Glucose-Positron Emission Tomography integrated with CT
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HLA	Human Leukocyte Antigens
IFN	Interferon
IL	Interleukin
irRC	Immune-related Response Criteria
IVIM	Intra Voxel Incoherent Motion
LM	Lentigo Maligna
LMM	Lentigo Maligna Melanoma
M-CSF	Macrophage-Colony Stimulating Factor (M-CSF)
MDSCs	Myeloid-Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MITF	Microphthalmia-associated Transcription Factor
MM	Malignant Melanoma
MRI	Magnetic Resonance Imaging
NK	Natural Killers
NKT	Natural Killers T-cells
OR	Objective Response
ORR	Overall Response Rate
OS	Overall Survival

PD	Progressive Disease
PD1/PDL1	Programmed Death receptor 1/Programmed Death receptor Ligand 1
PET/CT	Positron Emission Tomography/Computed Tomography
PFS	Progression Free Survival
RECIST	Response Evaluation Criteria in Solid Tumours
SNB	Sentinel Node Biopsy
STAT	Signal Transducers and Activators of Transcription
TAAAs	Tumour-Associated Antigens
TAMs	Tumour-Associated Macrophages
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
TILs	Tumour-Infiltrating Lymphocytes
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
Tregs	T regulatory cells
VEGF	Vascular Endothelial Growth Factor
UV	Uveal Melanoma

Introduction

Malignant melanoma

Background

Malignant melanoma (MM) is the most aggressive form of skin cancer. MM serves as a 'model' tumour for understanding cancer immunity [1]. The immune system is capable of spontaneously mounting a response against melanoma, which can be noted by signs of vitiligo and a complete regression of primary melanomas [2]. Up to 50% of primary melanomas undergo partial or complete spontaneous regression in the absence of all treatment or during a treatment that is inadequate to exert a significant influence on neoplastic disease [3]. In the process of regression, tumour-infiltrating CD4+ T-helper and CD8+ T-cytotoxic lymphocytes have been observed, possibly reflecting an efficient immune response against melanoma cells [4].

Melanoma was the first tumour type where tumour-associated antigens (TAAs) were identified and classified [5]. TAAs are recognized by autologous antibodies as well as T-cells, and can induce tumour-directed immune responses. Active antigen-specific immunotherapy was early investigated as a treatment option for advanced stage melanoma [6].

Various studies in melanoma have shown that the immune system can be manipulated to fight cancer, indicating that immunotherapy represents an effective therapeutic approach. Therefore, different types of immunotherapy for MM have been at the forefront of the immune-oncology research [1].

Epidemiology

Although melanoma accounts for only 4% of all skin cancers, it causes the greatest number of skin cancer-related deaths. In 2008, there were 84 000 new cases of cutaneous melanoma and 20 100 MM related deaths in Europe [7]. In Sweden, 3668 new cases of cutaneous melanoma, and 573 related deaths were reported during 2014. Despite prevention campaigns aimed at reducing excessive sun exposure, incidence rates are increasing at a faster rate compared to other cancers [8]. The most common risk factors for developing cutaneous melanoma are [9]:

- Ultraviolet light exposure
- Presence of different types of nevi
- Fair skin, freckling and light hair
- Family history of melanoma
- Personal history of melanoma or other skin cancers
- Immune deficiency
- Older age
- Male gender
- Xeroderma pigmentosum, a rare inherited condition that affects the skin cells' ability to repair DNA damage.

Pathogenesis

Melanocytes located in the basal layer of the epidermis form multiple contacts with keratinocytes. Keratinocytes stimulate different functions of melanocytes such as proliferation, differentiation, melanogenesis and dendritogenesis. Various keratinocyte-derived paracrine factors have a key role in regulating melanocyte function through receptor-mediated signalling pathways, followed by maintaining epidermal homeostasis [10].

Most melanomas arise within the epidermis escaping the control of keratinocytes. The development of MM from normal melanocytes is a complex procedure. Melanocytes can proliferate and form dysplastic nevi. The latter can be further developed to hyperplasia, invasion and metastasis. In this process numerous molecular events are taking place and many of them are the results of gene mutations.

Mutations of the NRAS or BRAF genes cause abnormal constitutive activation of the serine-threonine kinases in the extracellular signal-regulated kinase / mitogen-activated-protein kinase pathway (ERK/MAPK pathway) stimulating melanoma cell growth. Inhibition of NRAS and BRAF suppresses melanoma cell growth *in vitro* [11].

Mutations in two other genes, the CDKN2A- and PTEN-genes, can lead to development of melanoma. Abnormalities in the CDKN2A gene increase the probability that dysplastic nevi becomes a MM. Mutations in the CDKN2A gene hamper the recruitment of the alternate reading frame (ARF), a tumour-suppressor protein that arrests cell cycle and promotes cell death. PTEN-gene acts via phosphatidylinositol phosphate (PIP3) and AKT, a protein kinase B. Lack of PTEN leads to higher levels of PIP3 and AKT that in turn increases cell proliferation and prolongs cell survival.

Microphthalmia-associated transcription factor (MITF) regulates the development, differentiation as well as maintenance of melanocytes. Moreover, melanocyte pigmentation is also regulated by MITF. It is known that the process regarding the development from nevus to melanoma is accompanied by decreased or absent pigmentation. MITF amplification in MM is correlat-

ed with a poor prognosis. *In vitro*, overexpression of MITF and BRAF transforms primary melanocytes suggesting that MITF is an oncogene.

In addition, cadherins and integrins are of importance due to the fact that invasion and metastases of MM are the results of alterations in cell adhesion. Cadherins sustain cell-to-cell contacts, hence they provide the link with the actin cytoskeleton as well as induce signalling via P-catenin. Alterations in the expression of cadherins affect MM cell interaction with the environment and changes P-catenin signalling. Integrins mediate cell contact with various components of the extracellular matrix. MM growth is associated with the expression of $\alpha V\beta 3$ integrin that in turn increases the expression of the anti-apoptotic Bcl-2 [12, 13].

Finally, many tumour antigens have been identified on human melanomas such as the various MAGE genes (encoded by cancer-germline genes), Melan-A, gp100, TRp-2 and others encoded by differentiation genes as well as antigens resulting from point mutations [14].

The immune system of the patients with MM promotes a response with tumour-reactive T-cells but the response turns ineffective. This is most likely due to the development of local immunosuppression at the tumour sites.

Types of melanoma

Malignant melanoma can occur anywhere in the body.

I) Cutaneous melanoma

Melanoma of the skin. There are four basic types of skin melanoma based on the pathology report [10]:

Superficial spreading melanoma is the most common type (70 %). It grows along the top layer of the skin for a long time before penetrating more deeply. The microscopic hallmarks are: large melanocytic cells with nest formation along the dermoepidermal junction, invasion of the upper epidermis in a pagetoid fashion, invasion of the dermis by atypical, pleomorphic melanocytes and absence of the 'maturation' typical of nevus cells, with or without mitoses.

Lentigo maligna melanoma (LMM) is a melanoma that has evolved from a lentigo maligna (LM) and usually found on chronically sun-damaged skin. LM is the non-invasive skin growth that some pathologists consider to be a melanoma-in-situ. Once a LM becomes a LMM, it is treated as if it were an invasive melanoma

Acral lentiginous melanoma spreads superficially before penetrating more deeply. It usually appears under the nails or palms of the hands or the soles. Histological signs of acral lentiginous melanoma include: atypical melanocytes, dermal invasion and desmoplasia.

Nodular melanoma is usually invasive at the time of diagnosis. It is the most aggressive form of melanoma and it tends to grow more rapidly in

thickness than in diameter. The microscopic hallmarks are: dome-shaped at low power, epidermis thin or normal, dermal nodule of melanocytes with a 'pushing' growth pattern and no "radial growth phase".

II) Mucosal melanoma

Mucosal melanoma accounts for approximately 1 percent of all melanomas. The mucosal melanomas arise primarily in the head and neck, anorectal, and vulvovaginal regions. The prognosis is worse compared with cutaneous MM. Due to their rarity, their anatomic locations and their unique biology, the treatment options remain limited.

III) Ocular melanoma

Melanoma can rarely arise from melanocytes in the iris, the choroid layer and the conjunctiva of the eye. Two main subtypes of primary ocular melanoma have been described, uveal melanoma (UM) and conjunctival melanoma (CM).

Uveal melanoma's incidence is 5 per million making it the most common primary intraocular tumour in adults [15]. Uveal melanoma is genetically distinct from cutaneous melanoma, with 80% to 90% of UMs showing activating mutations in *GNAQ* or *GNA11* -genes encoding for G protein alpha subunits- and lacking activating mutations in *BRAF*, *NRAS* and *TERT* promoter [16]. Other driver mutations such as *CYSLTR2*, *PLCβ4*, *BAP1*, *EIF1AX*, and *SF3B1* have also been detected. Regarding pathogenesis of UM; different signalling networks are implicated, including the MAPK, PI3K, Hedgehog, YAP and apoptotic pathways.

Conjunctival melanomas represent approximately 5 percent of ocular melanomas. They arise within the conjunctiva: from a preexisting nevus or in areas of primary acquired melanosis or de novo [17].

Staging and risk assessment of cutaneous malignant melanoma

Physical examination with special attention to other suspicious pigmented lesions, tumour satellites, in-transit metastases, regional lymph node (LN) and systemic metastases is mandatory. In low-risk melanomas (pT1a) no other investigations are necessary. In tumour stages pT1b–pT3a ultrasound for locoregional LN metastasis may be considered and in high risk melanomas (pT3b, pT4) computed tomography (CT) or positron emission tomography/computed tomography (PET/CT) scans should be considered before surgical treatment and sentinel node biopsy.

The refined version of the American Joint Committee on Cancer (AJCC) staging and classification system, which includes sentinel node staging, is the only internationally accepted classification system for MM [18]:

TNM Staging Categories for Cutaneous Melanoma

Classification (T)	Thickness (mm)	Ulceration Status/Mitoses
Tis	NA	NA
T1	≤ 1.00	a: without ulceration and mitosis < 1/mm ² b: with ulcerations or mitoses ≥ 1/mm ²
T2	1.01-2.00	a. without ulceration b. with ulceration
T3	2.01-4.00	a. without ulceration b. with ulceration
T4	> 4.00	a. without ulceration b. with ulceration

N	No of met nodes	Nodal metastatic burden
N0	0	NA
N1	1	a. micrometastasis b. macrometastasis
N2	2-3	a. micrometastasis b. macrometastasis c. in transit metastases/satellites 'without' metastatic node
N3	≥4 met nodes, or matted nodes, or in transit met(s)/satellite(s) with metastatic node(s)	

M	Site	Serum LDH
M0	No distant metastasis	Normal
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

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a: Micrometastases are diagnosed after sentinel lymph node biopsy and completion lymphadenectomy

b: Macrometastases are defined as clinically detectable nodal metastases confirmed by therapeutic lymphadenectomy or when nodal metastasis exhibits gross extracapsular extension.

Stage grouping

Stage grouping is the process where T, N, and M groups are combined to give an overall stage, using Roman numerals I to IV. In general, patients with lower stage cancers have a better prognosis. Survival rates are often used regarding a patient's prognosis. Below shows the stage grouping and the disease specific survival rates by staging:

Stage IA: T1a, N0, M0, the 5-year survival rate is around 97% and the 10-year survival is around 95%.

Stage IB: T1b or T2a, N0, M0, the 5-year survival rate is around 92% and the 10-year survival is around 86%.

Stage IIA: T2b or T3a, N0, M0, the 5-year survival rate is around 81% and the 10-year survival is around 67%.

Stage IIB: T3b or T4a, N0, M0, the 5-year survival rate is around 70% and the 10-year survival is around 57%.

Stage IIC: T4b, N0, M0, the 5-year survival rate is around 53% and the 10-year survival is around 40%.

Stage IIIA: T1a to T4a, N1a or N2a, M0 The 5-year survival rate is around 78%. The 10-year survival is around 68%.

Stage IIIB: One of the following applies:

- T1b to T4b, N1a or N2a, M0
- T1a to T4a, N1b or N2b, M0
- T1a to T4a, N2c, M0

The 5-year survival rate is around 59% and the 10-year survival is around 43%.

Stage IIIC: One of the following applies:

- T1b to T4b, N1b or N2b, M0
- T1b to T4b, N2c, M0
- Any T, N3, M0

The 5-year survival rate is around 40% and the 10-year survival is around 24%.

Stage IV: Any T, any N, M1 (a,b,c). The 5-year survival rate is about 15% to 20% and the 10-year survival is about 10% to 15% when the spread is only to distant parts of the skin or distant lymph nodes. Once the spread is to other organs only 5% of the patients are alive after 5 years.

Treatment of cutaneous malignant melanoma

The treatment of choice for melanoma depends on disease stage and localisation. The patients' disease status will be characterised into one of the following three groups:

I) Primarily resectable

Early detection and excision of localized superficial cutaneous melanoma is considered to be the golden standard. Specific surgical margins are currently recommended for different subtypes of melanoma in order to prevent local recurrence in or around the scar [19].

Sentinel node biopsy (SNB) in melanoma with a thickness of >1 mm and/or ulceration is recommended for precise staging. It should be discussed in patients with a pT1b with a tumour thickness >0.75 mm. If the sentinel node is found positive for metastasis, a complete lymphadenectomy of the regional lymph nodes should be considered. This procedure gives an extended relapse-free survival without proven effect on overall survival (OS) [20].

Adjuvant therapies have been investigated. Adjuvant interferon (IFN) α -2b is the only adjuvant therapy approved by the US Food and Drug Administration for high-risk melanoma. However, no benefit related to OS has been demonstrated for adjuvant chemotherapy, nonspecific immunotherapy, radiation therapy, retinoid therapy, vitamin therapy, or biologic therapy. Immunotherapy and BRAF \pm MEK inhibitors (described below) are still experimental and should not be used outside controlled clinical trials [19]. However, two recently published phase III studies evaluated adjuvant therapy of patients with advanced MM. In a phase III study were 870 patients with complete resection of stage III BRAF-mutated melanoma assigned to receive dabrafenib + trametinib or placebo + placebo for a period of up to 1 year. This combination therapy resulted in a significantly longer recurrence-free survival and was not associated with new toxic effects [21]. In another phase III trial, 906 patients after complete resection of stage IIIB, IIIC, or IV melanoma, were randomized to receive either nivolumab (3 mg/kg) every 2 weeks or ipilimumab (10 mg/kg) every 3 weeks for four doses and then every 12 weeks for 12 months. Adjuvant therapy with nivolumab resulted in lower risk of recurrence with lower toxicity than adjuvant therapy with ipilimumab [22].

II) Locoregional recurrence or single distant metastasis

Surgical removal or stereotactic irradiation of locoregional recurrence or single distant metastasis should be considered in fit patients, offering a potential for long-term disease control [20].

III) Metastatic

Once a patient develops metastatic disease the prognosis is dismal [23]. Until recently, no agent has been shown to improve survival compared to best supportive care. During the last decade, immunotherapy and targeted therapy have emerged to the frontline. Below, some of the most commonly used therapies for metastatic disease are discussed.

Immunotherapy

The immune system plays an important role to combat cancer but cancer is a complex and dynamic tissue that develops different strategies to grow progressively and expand, which also includes immune escape [24]. The relationship between immunity and cancer has extensively been studied over the past decades [25] and is in depth discussed in the section “Tumour immunology”. Recently, immunotherapy has emerged as an established anticancer treatment in MM [26] while some are still experimental. Below, some of the most used strategies in the clinic are discussed.

The concept of cancer immunosurveillance was already proposed in 1970 [27]. It is now well established that the immune system is capable of spontaneously recognizing tumour antigens and developing a cytotoxic response by generating specific anti-tumour CD8+ T-lymphocytes in MM [28]. However, this anti-tumoural T-cell response eventually fails for two main reasons:

- Cancer immunoediting: a process that causes the elimination of cancer cells that express antigens recognized by T-cells [29, 30].
- Immune checkpoint pathways: the activation of immune suppressive pathways by the tumour that inhibit the initial anti-tumoural T-cell response.

Various strategies have been developed in order to assist the immune system, by blocking the inhibitory checkpoint molecules, thus enhancing the anti-tumour T-cell response.

The natural process, in which our immune system is kept in check to prevent autoimmune disease, may also hamper the development of an adequate anti-tumour response, or break an ongoing response. A major challenge is to develop approaches to allow priming or activation of T-cells in the tumour-bearing hosts [31].

The recent advances in the understanding of antigen presentation and tolerance have led to some promising immunotherapy strategies. Such strate-

gies include: vaccination, adoptive transfer of immune effectors and immunomodulatory therapy [32].

1) Adoptive cell transfer (ACT)

Adoptive cell therapy is a highly personalized anti-cancer therapy that involves administration of immune cells to the cancer-bearing host with direct anti-tumour activity. Tumour-infiltrating lymphocytes, *i.e* lymphocytes derived from resected tumours, are reinfused to the same patient after *in vitro* expansion. Interleukin (IL)-2 coinfusion has been used to enhance T-cell activation. Clinical trials have shown that the treatment of patients with disseminated MM with autologous tumour-infiltrating lymphocytes (TILs) and high dose IL-2 can be followed by objective responses (OR) reaching 50% and durable remissions [33]. Dudley et al performed a study where patients with metastatic MM were treated with intensive myeloablative chemoradiation preparative regimens. One group was treated with preparative lymphodepletion (cyclophosphamide 60 mg/kg/d for 2 days and fludarabine 25 mg/m²/d for the next 5 days) followed by 2 Gy total-body irradiation (TBI) and the other group by 12 Gy TBI. The 12 Gy TBI had better OR (72% versus 52%), however, more toxicity related adverse events than the 2 Gy TBI group [34].

Various preclinical experiments demonstrated the activation of an anti-tumour immune response by irradiation using doses higher than 5–20 Gy with the advent of stereotactic body radiotherapy (SBRT). It is therefore possible that irradiation and immunotherapy possess a synergistic effect depending on the dose given per fraction. In one trial, 12 patients with metastatic renal cell carcinoma or metastatic MM received one, two, or three doses of SBRT (20 Gy per fraction) with the last dose administered 3 days before starting IL-2. A minimum of one and a maximum of three lesions were treated with SBRT. All lesions treated with SBRT regressed and none have recurred. Overall response in non-irradiated target lesions were assessed by Response Evaluation Criteria in Solid Tumours (RECIST) criteria. A response rate of 67% compared to the historical 15% observed with IL-2 alone was observed [35].

A novel type of ACT concerns reinfusion of autologous peripheral blood T-cells genetically engineered to express chimeric antigen receptors (CAR) for specific tumour antigen recognition [36]. CAR T-cells are T-cells genetically engineered in order to express a tumour-targeting receptor. This tumour-targeting receptor is a chimera of a signalling domain of the T-cell receptor (TCR) complex and an antigen-recognizing domain. Hence, independently of the native TCR, CAR T-cells can recognize tumour cells via the CAR receptor. The CAR is not dependent on major histocompatibility complex (MHC). CAR T-cells get activated and proliferate *in vivo* upon contact with the antigen binding to. This can in turn lead to lysis of a large tumour burden and development of immunologic memory towards that specific tar-

get antigen [37]. Clinical studies with CD19-targeting CARs for the treatment of B-cell malignancy have shown positive results [38, 39]. In one recent study, 14 out of 20 patients with CD19⁺ chronic lymphocytic leukaemia achieved a complete response [40].

So far no well-established targets for melanoma-specific CAR T-cells have been defined, however, the patient's T-cells can instead be engineered with TCRs with high affinity for melanoma antigens. T-cells are transduced with a viral vector encoding a new antigen-specific TCR. An antigenic peptide is presented by Human Leukocyte Antigens (HLA) class I on the surface of the tumour cell. The receptor recognizes and interacts with the antigenic peptide-HLA class I complex leading to a T-cell-mediated cytotoxicity via induction of apoptosis [41]. The first successful clinical trial with TCR gene-engineered T-cells was reported in 2006. In this study fifteen patients with refractory disseminated MM were treated with infusion of autologous T-cells with a TCR against an HLA-A2-restricted epitope of MART-1. Two of these patients (13%) demonstrated objective clinical responses [42]. Since the above trial was performed various clinical trials followed with varying results. Patients with metastatic MM are currently recruited in studies involving the use of TCR-engineered T-cells.

Even if ACT approaches have been used to treat MM patients for many years, few clinics have had the capacity to establish such *ex vivo* expansion protocols and neither of these treatments are yet approved therapeutics by the regulatory bodies.

2) Immune checkpoint inhibitors

Several antibodies have been approved for MM during the past few years. All of them target so-called T-cell checkpoint pathways.

Cytotoxic T-lymphocyte antigen 4 (CTLA4) is a member of the CD28:B7 immunoglobulin superfamily expressed at low levels on the surface of naive effector T-cells and at a higher level, on T regulatory cells (Tregs). Naive T-cells recognize, through their TCR, antigens presented by the major histocompatibility complex (MHC) on the surface of cancer cells. After the T-cell activation, CTLA4 localizes to the plasma membrane and competes with CD28 for B7. This process leads in turn to the upregulation of CTLA4 and through a variety of mechanisms turns off T-cell receptor signalling and subsequently negatively regulates T-cells. CTLA4 serves as a physiologic “brake” on the activated immune system in order to maintain homeostasis [43].

Programmed cell death 1 (PD1) is a trans-membrane protein expressed on activated CD4⁺ and CD8⁺ T-cells, B-cells, natural killers T- (NKT) cells and monocytes. PD1 has two ligands, programmed cell death L1 (PDL1) and programmed cell death L2 (PDL2), which are members of the B7 family [44]. PDL1 is located on the surface of tumour cells and myeloid cells [45]. PD1 and PDL1 are immune down-regulators or immune checkpoint “off

switches” [46]. The interaction between PD1 and PDL1 mediates peripheral tolerance due to suppression of T-cell activity and reduction of T-cell-mediated cytotoxicity [47]. Specifically, this interaction leads to the inhibition of effector T-cells and thus immune evasion by cancer cells.

Antibodies targeting CTLA4, PD1 or PDL1 are designed to block these inhibitory signals preventing inhibition of T-cells thereby enhancing anti-tumour activity. These antibodies are collectively called “immune checkpoint blockers”.

Ipilimumab (Yervoy[®]) is an antagonistic monoclonal antibody directed against CTLA4. Thru this the therapy amplifies T-cell activation and proliferation and thereby enhances the anti-tumour immune response. Ipilimumab significantly improved OS in pre-treated and previously untreated patients.

In a clinical study, of patients with previously untreated metastatic MM, one group was treated with ipilimumab+dacarbazine and the other with dacarbazine+placebo. OS was significantly longer in the group receiving ipilimumab+dacarbazine (11.2 months vs. 9.1 months, with higher survival rates in the ipilimumab-dacarbazine group at 1 year (47.3% vs. 36.3%), 2 years (28.5% vs. 17.9%), and 3 years (20.8% vs. 12.2%) [48].

In another study with three groups (ipilimumab alone, gp100 alone and ipilimumab plus gp) of patients with metastatic MM, the median OS was 10.0 months for patients with ipilimumab + gp100, as compared with 6.4 months with gp100 alone. The median OS with ipilimumab alone was 10.1 months [49]. Based on these results, ipilimumab was approved for the treatment of patients with metastatic melanoma.

Pembrolizumab (Keytruda[®]) and *nivolumab* (Opdivo[®]) are monoclonal antibodies binding to and blocking PD1 receptor. The PD1/PDL1 pathway is a negative regulator of T-cell proliferation and cytokine production. Therefore, blocking of PD1/PDL1 increases the T-cell activation and subsequently eliminates tumour cells.

In the KEYNOTE-002 study 540 patients with ipilimumab-refractory MM were enrolled. 180 patients were randomly assigned to receive pembrolizumab 2 mg/kg (group 1), 181 to receive pembrolizumab 10 mg/kg (group 2), and 179 to receive chemotherapy (group 3). A 6-month progression free survival (PFS) was observed in 34% of patients in group 1, in 38% in group 2 and in 16% in group 3 [50].

The combination of anti-CTLA4 and anti-PD1 antibodies was tested in advanced stage melanoma. The patients who were treated with the combination of ipilimumab and nivolumab experienced a higher objective response rate in comparison to standard treatment with nivolumab monotherapy [51, 52]. Larkin et al showed that the median PFS was 11.5 months for patients with untreated MM who were treated with nivolumab + ipilimumab, as compared with 2.9 months with ipilimumab and 6.9 months with nivolumab [53]. CTLA4 and PD1 regulate distinct inhibitory pathways in T-cells [54,

55]. Blocking of CTLA4 leads to an increase of T-cells in the tumour and anti-PD1 overcomes the immunosuppressive microenvironment. CTLA4 and PD1 seem to synergize in eliciting an immunogenic microenvironment [56]. FDA approved this combination in 2015.

3) Oncolytic virus therapy

Viruses can replicate in cells and cause cell death during the lysis process in which new virus particles are released from the infected cell. This process can be controlled to occur in cancer cells by restricting the replication process. For example, the transcription of viral genes important for replication can be under the control of promoters present in only tumour cells or certain tissues. Such genetically engineered viruses are called oncolytic viruses. Lately, such viruses have been further enhanced with immunostimulatory genes to achieve both tumour cell oncolysis and immune activation [57].

Talimogene laherparepvec (T-VEC) (Imlygic[®]) is an oncolytic virus derived from herpes simplex virus type-1. It is designed to selectively replicate within tumours and to produce Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) in order to enhance systemic anti-tumour immune responses. Imlygic[®] is given as a series of intratumourally injections in patients with unresected melanoma [58].

Encouraging data from early Phase I and II trials testing intratumoural T-VEC in patients with various tumours led to various phase III clinical studies, including OPTiM (OncoVEX^{GM-CSF} Pivotal Trial in Melanoma) [59, 60]. In OPTiM trial, over 400 patients with Stage IIIB-IV melanoma with injectable but not resectable tumours were randomized (at a 2:1 ratio) between intralesional T-VEC or subcutaneous GM-CSF [61]. This trial, confirmed the superiority of intralesional T-VEC: durable response rate (16.3% vs. 2.1%, $p < 0.001$) and overall response rate (ORR) (26.4% vs. 5.7%, $p < 0.001$) were significantly better while median OS was not significantly better for patients receiving T-VEC (23.3 vs. 18.9 months, $p = 0.051$). The most common adverse events related to the administration of T-VEC were fatigue, chills, and pyrexia. The only severe toxicity (in $> 2\%$ of patients) reported was cellulitis [62]. T-VEC was approved by FDA for the treatment of patients with advanced MM without visceral metastases and with \geq one injectable metastasis.

4) Vaccines

There is a long history of attempting to harness the adaptive immune recognition of a cancer-related antigen to elicit anti-tumour responses. Antigen choices range from simple peptides to whole cell preparations [63].

Sipuleucel-T is the only approved vaccine-based therapy for advanced cancer. It is an autologous dendritic-cell preparation engineered to target prostatic acid phosphatase. In a randomized phase III trial were enrolled 512 patients with castrate-resistant prostate adenocarcinoma. 341 patients re-

ceived the sipuleucel-T and experienced a 4-month improvement in median survival [64].

In immunogenic cancers, as melanoma, various single-peptide vaccines continue to be tested. All these studies have shown disappointing efficacy in both preventing recurrence or prolonging survival. In one randomized phase III trial 185 patients were enrolled and received either a combination of IL-2 plus the HLA-A*0201- MHC-specific vaccine against the surface glycoprotein gp100 or IL-2 alone. Patients who received the combination had a higher response than those who received IL-2 alone (22 versus 10 percent). Furthermore, a non-significant trend toward improved survival was observed (17 versus 11 months) [65]. Nevertheless, in the randomized clinical trial that demonstrated a survival benefit for ipilimumab with or without this gp100 vaccine, the vaccine did not improve survival over ipilimumab alone [49].

In a contemporary study, three patients with MM had their resected tumours sequenced for somatic alterations that were predicted to bind with high affinity to their own HLA-A*02-01 MHC molecules. Seven candidate peptides were injected into each patient, and 9 out of the 21 peptides led to immune responses suggesting that patient-specific vaccination approaches may be feasible [66].

New targets for immunotherapy including new vaccine strategies are currently under development. A few examples are: the tumour necrosis receptor (TNRF) superfamily, cell surface proteins within the immunoglobulin superfamily (IgSF), B7 and CD28-related proteins. Furthermore, clinical trials with indoleamine 2,3-dioxygenase (IDO) inhibitors in combination with a PD1 inhibitor for metastatic melanoma are ongoing [67, 68].

Targeted therapy

Another approach for the effective treatment of MM is targeted inhibition of key mechanistic events such as cell proliferation and survival, angiogenesis and invasion or metastasis.

The MAPK pathway holds an important role in the development of MM making this cascade an interesting therapeutic target. However, identification of the ideal pathway member for maximal clinical benefit remains a challenge. In normal cells, the MAPK pathway relays extracellular signals from the cell membrane to the nucleus via a cascade of phosphorylation events that promote cancer development. Dysregulation of this signalling cascade occurs frequently in MM. Genetic or epigenetic modifications in the BRAF, RAS and MEK genes are the key aberrations observed in the MAPK pathway. Combined inhibition may be required to prevent the progression and development of resistance in this disease [69].

1) BRAF-inhibitors (Vemurafenib - Zelboraf[®], Dabrafanib - Tafinlar[®])

The BRAF-gene, which is the most frequently mutated gene in the MAPK pathway, is actively mutant in over than 60% of advanced melanomas. A change of valine to glutamic acid at codon 600 (V600E) in exon 15 is prevalent in 90% of the BRAF-mutations [70]. BRAF^{V600E} leads to hyperactivation of the MAPK pathway, which in turn triggers survival pathways and cell division to promote tumour development by inducing proliferation [71]. Occurrence of BRAF mutation is likely an early event whereas the alteration of the PTEN/AKT pathway occurs later in tumour progression [72]. In large clinical trials, small-molecule inhibitors, such as vemurafenib and dabrafenib, targeting mutated BRAF kinase have shown significant efficacy, including prolonged survival. Major concerns related to BRAF-inhibitors include development of resistance and to a lesser extent drug-related side effects [73] (see below).

In the last decade the effects of BRAF-inhibitors on cells of the immune system and on anti-tumour immunity have been studied. Although BRAF-inhibitors are not designed to directly activate anti-tumour immune responses, there is evidence of enhanced anti-tumour immunity and correlation with clinical responses. In *in vivo* studies with BRAF^{V600E} melanoma tumours the presence of immunoregulatory cell subsets [such as Tregs and myeloid derived suppressor cells (MDSCs)], fewer CD4 T-cells producing IFN γ , tumour necrosis factor (TNF) α (TNF α) and IL2 as well as lower expression of CD40L were observed. Compromised CD40/CD40L signalling leads to loss of maturation signals that are necessary for antigen presentation. This increases in turn the concentration of immature DCs and macrophages in tumours. BRAF^{V600E} inhibitor treatment not only reduces tumour growth but also increases CD4+ and CD8+ T-cell infiltration. Studies suggest that inhibition of the BRAF-MAPK pathway may influence host immune responses in the tumour microenvironment (TME) as well as systemically. These effects may be related to reduced tumour cell viability and reduced tumour-induced immunomodulation. The resistance to BRAF-inhibitors as well as the tumour-induced immunosuppressive equilibrium at the time of progression of the disease may indicate a link between mutant BRAF dysregulation and immune signals alterations in cancer [74].

2) MEK-inhibitors (Cobimetinib - Cotellic[®], Trametinib - Mekinist[®])

MEK-1 and MEK-2 are dual-specific tyrosine/threonine protein kinases that lie downstream of BRAF. They are active in ~30% of all human cancers with activated MAPK signalling. MEK-1/2 is an interesting therapeutic target in the MAPK cascade [75]. BRAF mutation status is a critical factor since tumours that harbour BRAF^{V600E} are sensitive to MEK inhibition, however, not those with mutant RAS [76].

In a phase 3 open-label trial Flaherty et al assigned 322 patients who had metastatic MM with a BRAF mutation to receive either trametinib, an oral

selective MEK inhibitor, or chemotherapy in a 2:1 ratio. Median PFS was significantly increased with trametinib compared to chemotherapy (4.8 months in the trametinib group versus 1.5 months in the chemotherapy group). Six month OS was significantly improved with trametinib compared to chemotherapy (81 versus 67 percent), even though 47% of patients who experienced disease progression on chemotherapy received secondary treatment with trametinib [73].

Certain melanoma cells are resistant to MEK1/2 inhibitors despite the presence of BRAF mutation but the mechanisms leading to this resistance remain unclear [77]. By targeting multiple points in the MAPK signalling preventing MEK mediated resistance may be possible. The combination of MEK- and BRAF-inhibitors in BRAF-mutated melanoma cells are tested in order to prevent development of resistance [78] (Figure 1).

In a phase 3 trial, 423 previously untreated patients who had unresectable stage IIIC or stage IV MM with a BRAF mutation were randomly assigned to receive a combination of dabrafenib + trametinib or dabrafenib + placebo. The median PFS was significantly prolonged with the combination compared with dabrafenib alone (11 months in the dabrafenib + trametinib group versus 8.8 months in the dabrafenib + placebo group). The ORR was 67% in the dabrafenib + trametinib group and 51% in the dabrafenib + placebo group. Overall survival was improved with the combination (median 25.1 versus 18.7 months). The improvement in OS was seen despite the fact that more patients assigned to dabrafenib alone subsequently received additional systemic therapy [79].

In the other phase III trial, 704 patients with previously untreated metastatic melanoma and BRAF^{V600} mutation were randomly assigned to either dabrafenib + trametinib or vemurafenib. The trial was stopped for efficacy based upon positive results after a planned interim analysis. Overall survival was significantly increased with the dabrafenib + trametinib combination (one-year survival rate 72 versus 65 %). Median PFS was also significantly increased (11.4 versus 7.3 months) [80].

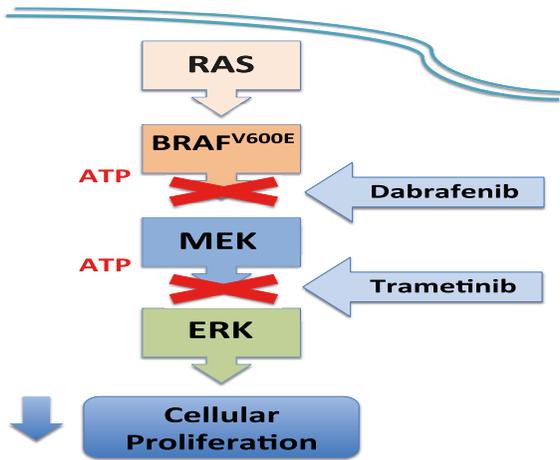


Figure 1 The intracellular RAS/RAF/MEK/ERK signalling cascade

3) KIT inhibitors

KIT mutations are present in 10% of melanomas, especially in MM that arise from mucosal or acral lentiginous surfaces [81]. In three different phase II trials imatinib, a kinase inhibitor, has proven efficacy in patients with advanced MM harbouring KIT mutation. In these three trials the OR was 16-30%, PFS was 3-4 months and median OS was 10.6-14 months. The adverse events were mild to moderate. The most common side effects were oedema, fatigue, nausea, neutropenia, and elevated transaminases. Certain mutations in exon 11 and 13 of c-KIT were associated with the highest response rate [82-84]. Similar results were observed in trials with nilotinib, a tyrosine kinase inhibitor [85, 86]. Thus, sensitivity to KIT inhibition exists in a small subpopulation of metastatic MM.

Treatment of metastatic ocular melanoma

Uveal melanoma

Almost half of the patients with UM will eventually develop metastases and then the prognosis is quite poor with a median survival of 1 year [87].

It is well known that the eye is an immune-privileged organ, however, inflammation can still be present in the intraocular tumour. Several studies confirmed that the inflammatory phenotype in UM is characterized by an increased number of macrophages, TILs, as well as an increased expression of HLA of both class I and II on UM cells [88]. In contrast with the majority of other malignancies, in UM higher expression of the HLA class I and II worsens the prognosis [89]. Furthermore, the sensitivity of UM to natural

killer (NK)-cell mediated lysis is inversely correlated to HLA expression [90]. Several studies indicate that the presence of an infiltrate is a bad prognostic factor in UM. UM contains FoxP3-positive Tregs, which can lead to the inhibition of an effective cytotoxic T-lymphocyte (CTL) function [91]. A specific deletion of these Tregs could be a therapeutic approach since it may also reduce the number of the pro-angiogenic M2-macrophages and subsequently influence the development of the tumours [92]. New studies suggest that UM has a functioning HLA expression system that allows T-cell-mediated tumour cell killing. Hence, T-cell-based therapies should be effective in UM [93].

Studies of adjuvant therapy did not demonstrate a survival benefit in patients with high risk of metastases [94]. Dendritic cell (DC)-based immunotherapy has shown promising results in cutaneous melanoma patients [95]. Cutaneous melanoma and UM share many tumour antigens, although lower in UM, providing a base for the application of DC-based therapies in UM. Bol et al enrolled 14 patients with metastatic MM and showed that DC-vaccination was safe and effectively induced immune responses in patients. Tumour-specific CD8⁺ T-cells were detected in 30% of patients. Interestingly, the median OS was 19.2 months. It is pointed out, however, that this vaccination might possess a more profound role as an adjuvant treatment [96].

The predilection for liver metastases observed in UM could be a consequence of the activation of various downstream signalling pathways as a result of the over expression of molecules such as HGF, IGF-I and CXCL12. However the exact mechanism is not yet fully understood [97]. Since it is almost always a question of haematogenous dissemination with high frequency of hepatic involvement, post local treatment follow-up with liver imaging is the gold standard [98, 99]. Data are limited regarding systemic therapy in patients with advanced UM. Among patients without extrahepatic disease, better responses have been described if regional liver treatment is performed. Liver metastasectomy in carefully selected patients can result in improvement in OS [100]. Neither hepatic artery infusion with fotemustine and melphalan, nor transarterial chemoembolization with different chemotherapy agents led to improvement in OS. However, they may result in longer PFS or stabilization of hepatic metastases [101, 102]. Chemotherapy administered intravenously has no impact on survival [103].

There is only weak evidence that checkpoint inhibitor immunotherapy may have a place in the treatment of metastatic UM. In a phase II trial with ipilimumab (3 mg/kg), 45 pre-treated and 8 treatment-naïve patients were enrolled. The median OS was 6.8 months and the median PFS was 2.8 months [104]. A retrospective analysis of 56 cases of patients with metastatic UM who were treated with anti-programmed cell death protein 1 (PD1) antibodies suggests that the benefit was at most modest. Only 3.6% of the patients had partial response and 8.9% experienced stable disease. Median PFS was 2.8 months while OS was 7.6 months [105]. Preliminary evidence

from an ongoing phase 2 study indicates that patients who were treated with lymphodepleting conditioning cyclophosphamide (60mg/kg) for 2 days followed by fludarabine (25mg/m²) for 5 days and then received autologous TILs and high-dose interleukin-2 could benefit from regression of UM metastases [106].

Studies with molecularly targeted agents have shown very promising results. MAPK pathway is activated in UM and is considered a potential therapeutic target. In a randomized phase II study, 99 patients with advanced UM, without prior treatment with chemotherapy, were treated either with the MEK inhibitor selumetinib or chemotherapy (temozolomide or dacarbazine). Almost all patients had M1c disease, predominantly with liver metastases, and none had received prior temozolomide or dacarbazine. Treatment with selumetinib significantly prolonged PFS compared with chemotherapy (median 15.9 versus 7 weeks). Median OS was increased with selumetinib compared with chemotherapy, although not statistically significant (median 11.8 versus 9.1 months) [107]. Preliminary data of the SUMIT trial – a phase 3 study that randomized patients with metastatic UM to dacarbazine + selumetinib vs dacarbazine + placebo show no difference in PFS [108].

Conjunctival melanoma

Regional lymph node metastases are commonly present in patients with CM, whereas metastases in parotid nodes are most common [109].

In contrast to UM, CM is a form of mucosal melanoma. Since the molecular pathogenesis of CM appears to be more similar to that of cutaneous melanoma than to UM, the management of metastatic CM should be the same as for metastatic cutaneous melanoma.

However, both UM and CM express high levels of the differentiation antigens (*e.g* gp100, Melan-A etc) [110] but low levels of cancer/testis antigens, compared with cutaneous melanoma. These low levels suggest that immunotherapy directly targeting these antigens may not be effective for ocular melanoma [111].

No current therapy for advanced UM and CM has succeeded to improve OS of patients, highlighting the urgent need for new therapeutic strategies.

Side effects of immunotherapy and targeted therapy

Although immunotherapy holds much promise for the treatment of melanoma, it is important to note that it is associated with autoimmune side effects. Regarding ipilimumab side effects include colitis, bowel perforation, hepatitis, skin reaction, neurological problems, hormone gland and ophthalmological problems. For PD1 inhibitors the most common side effects are fatigue, nausea, rash, constipation, arthralgia, and diarrhoea whereas serious side effects are pneumonitis, colitis, hepatitis, nephritis, kidney failure, endocrine dysfunction, anaemia and severe muscle weakness.

Immune-mediated adverse effects related to ipilimumab can vary from mild to severe. Mild adverse effects can be managed with oral antihistamines or topical steroids while severe adverse events require systemic use of steroids or discontinuance of ipilimumab. In addition, treatment with infliximab, an antibody against TNF α generally used to treat autoimmune disease, can be considered for patients with severe or life-threatening immune-mediated toxicity who do not respond adequately to steroids.

The frequency of immune-mediated adverse events with PD1-directed therapy is lower than with CTLA4-blocking antibodies. The management of those adverse events is the same as for ipilimumab-related adverse events (see above) [112].

Common side effects for BRAF- and MEK- inhibitors are rash, cutaneous squamous cell carcinoma, diarrhoea, pyrexia, arthralgia, fatigue, cardiac problems, pneumonitis, ophthalmological problems, hypertension, liver laboratory abnormalities and hyperglycemia.

Life-threatening toxicities associated with BRAF- and MEK-inhibitors toxicities are extremely rare. The treatment should be continued in the presence of mild toxicities and other pharmacological agents could be considered to assist in improvement of the symptoms. Moderate and severe toxicities warrant treatment interruption and retreatment should be considered once the reaction has resolved. Standard dose reductions are recommended although it may be possible to maintain a dose by managing toxicities with other interventions [113].

Finding a balance between the effectiveness of different immunotherapies as well as targeted therapies and the incidence of treatment-related adverse events is therefore an important consideration. Furthermore, many questions remain unanswered regarding the optimal use of immunotherapy and targeted therapy. Obviously, melanoma will remain at the cutting edge of research since there is wide potential for improvement and optimization of both immunotherapy and targeted therapy.

Treatment recommendations

The Swedish guidelines are evidence-based and in accordance with the European guideline. In localized disease, wide excision of primary tumours is recommended. SNB in high and intermediate risk melanomas is recommended in order to determine staging. SNB-positive patients should be considered as candidates for a complete lymphadenectomy of regional lymph nodes. Therapeutic lymphadenectomy for clinically advanced, high-risk, lymph node-metastatic melanoma (*i.e* palpable lymph node metastases, radiological lymph node enlargement) improves OS [19].

Malignant melanoma is often considered to be a radio-resistant tumour. Various studies have shown that adjuvant radiation therapy (RT) provides good regional control and may have an impact on disease-specific survival [114]. However, toxicity is not negligible. This therapy should be considered

only for patients with poor anatomopathological features such as palpable lymph node metastases, radiological lymph node enlargement, extracapsular extension etc [115].

Subsequently, radiotherapy 2.5 Gy daily to 47.5 Gy or 6 Gy twice a week to 30-36 Gy should be considered in the following cases:

- Doubtful radical resection after attempt to radical lymphadenectomy
- \geq four lymph node metastases
- Lymph node metastases \geq 3 cm
- Extracapsular extension

Patients with metastatic melanoma should be screened for detection of BRAF^{V600} mutation in the primary tumour or metastases. Treatment options for the first- and second-line include anti-PD1 antibodies, (more effective than ipilimumab, see above) regardless of BRAF-mutational status, and BRAF/MEK-inhibitor combinations for patients with BRAF-mutant melanoma. If clinical trials or the approved new targeted compounds are not available, cytotoxic drugs such as dacarbazine or temozolomide with only modest activity may be administered.

Pregnancy and treatment with the new agents

With the increasing use of modern biologic agents, questions frequently arise in clinical practice regarding the safety of treatment in pregnant women. Data is very limited because for ethical reasons pregnant patients are not enrolled in approval studies. *In vitro* models, however, do not reflect any systemic repair mechanisms. In addition, due to the species specificity of antibodies, standard animal studies are not possible in the case of biologics. Thus, it is necessary to resort to analogous, for instance murine, antibodies with the same target structure or testing of high doses on cynomolgus monkeys [116].

Vemurafenib: In *in vivo* experiments, the drug has been shown to cross the placenta, however not displaying any teratogenic effects. There is currently no clinical data available on vemurafenib and its use during pregnancy. Paper IV is to our knowledge the only report documenting the administration of vemurafenib for MM during pregnancy [117].

Dabrafenib: There is no clinical experience for the use of dabrafenib during pregnancy. In *in vivo* experiments, the number of corpora lutea was reduced. Dabrafenib is teratogenic and affects embryonic/fetal development.

Ipilimumab, pemprolizumab and nivolumab: To date, there has been no clinical experience with the use of the above-mentioned antibodies in pregnant women. Ipilimumab (IgG1) and pemprolizumab (IgG4) as well as nivolumab (IgG2) cross the placental barrier. In *in vivo* experiments, treatment with all three antibodies separately led to a dose-dependent increase in miscarriages, stillbirths and premature births. The mortality rate among the offspring was increased. Therefore, their use is not recommended during pregnancy.

Radiological response evaluation

Assessment of the change in tumour burden is an important feature of the clinical evaluation of cancer therapeutics. To avoid continuing inefficient anticancer treatment, the accurate assessment of therapeutic response is essential. A tool was needed to standardize and simplify response criteria. Response criteria known as RECIST were established in 2000 and were revised a few years later (RECIST 1.1) [118]. RECIST criteria include the definitions of minimum size of measurable lesions, instructions on the number of lesions to follow, assessment of pathological lymph nodes, the use of unidimensional measures for overall evaluation of tumour burden.

While chemotherapies and targeted therapies induce direct cytotoxic effects on cancer cells, immunotherapies target the immune system and exert indirect effects on tumours with distinct characteristics in the clinic that are not necessarily reflected during radiologic evaluation. However, CT still remains the “gold standard” for staging as well as monitoring treatment response in MM patients [119]. An interesting question is whether it is time to move from anatomic unidimensional evaluation of tumour burden to either functional and/or volumetric anatomical assessment with positron emission tomography (PET) or magnetic resonance imaging (MRI). 18F-fluorodeoxyglucose (FDG)- PET integrated with CT (PET/CT) and MRI are increasingly used.

Until now the use of FDG-PET imaging is considered just an adjunct to determination of disease progression [120]. FDG-PET/CT is used for monitoring of various types of cancer [121-123] but is expensive and with the disadvantage that it sometimes is difficult to differ the actual tumour from inflammation induced by surgery, radiation therapy or an infection [119].

Immune-related response criteria (irRC) are a useful tool to evaluate responses to immunotherapy. The concepts for identifying certain immunotherapy response patterns include: (i) image-based confirmation of progression via subsequent scan(s), (ii) accept durable stable disease as a benefit and (iii) treating beyond conventional progression if the clinical situation allows [124].

The irRC criteria are recommended for the evaluation of immune therapies to ensure assessment of activity based on clinically relevant criteria and time points. With the new immune therapies, an immune mediated increase in tumour burden including the appearance of new lesions before radiographic responses are evident necessitates appropriate follow-up at a subsequent time point to confirm progressive disease (PD). Treatment should be continued as tumours may begin to shrink in this interval. Patients who are treated with immune therapy should undergo the first evaluation at week 12 after initiation of the treatment and thereafter every 3 months. If the first evaluation at week 12 shows PD, a second scan should be performed one

month later in order to confirm or reject that the treatment is ineffective [125, 126].

The use of diffusion-weighted - magnetic resonance imaging (DW-MRI) is increasingly being included in the assessment of tumour response for various cancers but is still limited to research settings. DW-MRI has applicability for cancer diagnosis, to differ benign from malignant lesions and to assess recurrent disease as well as treatment response [127, 128]. DW-MRI is a form of imaging method that is based on the random movement of water molecules mostly in the extracellular space. This movement is restricted in hypercellular tissues (*e.g.* in tumours), resulting in lower values of the so called apparent diffusion coefficient (ADC) compared with normal tissues [129]. The ADC is calculated by using the mono-exponential decay of signals [130]. An increase of ADC is thought to arise from tumour lysis, loss of cellular density, and increased extracellular space. This ADC increase probably indicates necrosis achieved by effective anticancer treatment [128].

However, the DWI signal is also influenced by pseudorandom motion in the capillary network. Therefore, the intra voxel incoherent motion (IVIM) model, which is a refined analysis technique to separate perfusion- and diffusion-related effects by assuming biexponential behaviour of signal decay, is increasingly employed [130-132]. The IVIM-DWI technique can measure both true molecular diffusion in tissues and perfusion in the capillary network and in that way improve the assessment of malignant lesions. True diffusion coefficient (D), perfusion-related coefficient (D^*) and perfusion fraction (f) constitute the IVIM-parameters. According to animal studies, changes in IVIM perfusion parameters could be useful for early tumour response assessment [133].

Tumour immunology

The immune system is a network of cells, tissues and organs that helps the body against potential attacks of “foreign”/non-self antigens. The two components of the immune system are the innate and the adaptive immune system.

The innate, also known as the nonspecific immune system, defends the host from infection by other organisms. The cells of the innate system recognize and respond to pathogens in a generic way, however, the system does not confer long-lasting or protective immunity to the host. The major functions of the innate immune system include [134]:

- Recruiting immune cells to the sites of infection, through the production of chemical factors, including specialized chemical mediators, called cytokines.
- Activation of the complement cascade to identify bacteria, activate cells, and promote clearance of antibody complexes or dead cells. The complement system is a biochemical cascade that attacks the surfaces of foreign

cells. It contains over 20 different proteins and has the ability to "complement" the killing of pathogens by antibodies.

- Identification and removal of foreign substances present in organs, tissues, blood and lymph, by the innate leukocytes such as phagocytes (macrophages, neutrophils and DCs), mast cells, eosinophils, basophils and natural killers (NK)-cells.
- Activation of the immune system through a process known as antigen presentation (more details in section "The cancer immunity cycle").

The adaptive immune response is antigen-specific and requires the recognition of specific antigens during antigen presentation. Antigen specificity allows the generation of responses that are tailored to specific pathogens or pathogen-infected cells. The cells of the adaptive immune system are special types of leukocytes, called lymphocytes. B-cells and T-cells (the T-cells further divided into CD4+T and CD8+T-cells) are the major types of lymphocytes and they are derived from hematopoietic stem cells in the bone marrow. B-cells and their antibodies constitute the humoral immune system, whereas T-cells are responsible for the cell-mediated immune response [135].

The immune system has a great potential for the specific destruction of tumours. Tumours are recognized by the immune system and their development can be disturbed through a process known as immunosurveillance. Tumour specificity of the immune response depends on the recognition of tumour antigens. In many cancers the malignant progression is accompanied by profound immune suppression that interferes with an effective anti-tumour response and tumour elimination. This immune suppression comes from the ability of tumours to subvert normal immune regulation to their advantage.

Tumour microenvironment

Tumours consist not only of the malignant cancer cells, but also of stroma cells that support the TME and other non-malignant cells. The tumour stroma basically consists of specialized mesenchymal cell types, innate and adaptive immune cells, vasculature with endothelial cells and pericytes as well as the extracellular matrix consisting of structural proteins, specialized proteins and proteoglycans. Intercellular communication occurs via the secretion of cytokines, chemokines, growth factors as well as inflammatory and matrix remodelling enzymes that act in autocrine and/or paracrine manners to support/sustain tumour development [136]. The main cells of the TME are: T- and B- lymphocytes, NK- and NKT-cells, tumour-associated macrophages (TAMs), MDSCs, DCs, tumour-associated neutrophils, cancer-associated fibroblasts, adipocytes, vascular endothelial cells, pericytes and lymphatic endothelial cells.

The presence in TME of a subgroup of T-lymphocytes, $\gamma\delta$ T-lymphocytes, has been described. This subgroup possesses mainly characteristics of innate immune cells with a cytotoxic activity against a wide range of malignant cells [137, 138]. It is still uncertain whether their presence is associated to a good or bad prognosis. Tumour-associated macrophages are abundant in most human cancers, exerting pro-tumorigenic activities and are associated with poor prognosis [139]. One of the most important roles of TAMs is their contribution to tumour angiogenesis [140]. Through the interaction between macrophages and TME, the phenotype of the macrophages is shaped. Evidence suggests that TAMs accumulate in hypoxic and/or necrotic areas of tumours since these areas release hypoxia-induced chemoattractants, such as vascular endothelial growth factor (VEGF) and endothelins [141]. The contribution of tumour-associated neutrophils (TANs) to tumour growth and metastasis is still controversial.

The tumour microenvironment can prevent the expansion of tumour antigen-specific CD4+ and CD8+ T-cells and instead promote the production of pro-inflammatory cytokines and other factors, leading to the accumulation of suppressive cell populations that instead inhibit the immunity, such as Tregs and MDSCs.

Immunosurveillance

Over the past decades the concept that the immune system can indeed control cancer has been studied. Already in the 1950s Burned and Thomas propose the so called cancer immunosurveillance theory [27]. The theory proposed that adaptive immunity was capable of preventing tumour development in immunocompetent individuals. In the 1990s, additional evidence supporting this theory was demonstrated [142]. According to the immunosurveillance theory, cancer cells express antigens that differentiate them from the normal cells and can thus be recognized by the immune system as “foreign” [30]. NK- and T-cells are the most important effector cells involved in anti-cancer responses.

The “cancer immunity cycle”

Chen et al proposed the cancer-immunity cycle that describes in detail how specific anti-cancer T-cell immune responses are elicited [26]. The cancer-immunity cycle can be divided into seven major steps, starting with the release of antigens from the cancer cells and ending with the effective killing of cancer cells. Each step of the cycle requires the coordination of numerous factors, both stimulatory and inhibitory. Stimulatory factors promote immunity, whereas inhibitors keep the process in check and reduce immune activity and/or prevent autoimmunity.

In the first step, antigens are released from dead cancer cells and are captured by antigen presenting cells (APCs) called DCs for processing. In order for this step to yield an anti-cancer T-cell response, it must be accompanied by signals that induce a simultaneous activation and migration. Such immunogenic signals might include pro-inflammatory cytokines and factors released by dying tumour cells as well as receptor/ligand interactions.

In the second step, DCs process the antigens and present them on MHC class I to CD8+ T-cells and MHC class II molecules to CD4+ T-cells in regional lymph nodes. The ability of DCs to regulate immunity is dependent on DC maturation. A variety of factors can induce maturation including: whole bacteria or bacterial-derived antigens, inflammatory cytokines, ligation of select cell surface receptors *e.g.* CD40, viral products, signalling through toll-like receptors (TLRs) [143]. During their conversion from immature to mature cells, DCs undergo a number of phenotypical and functional changes. The process of DC maturation involves:

- a redistribution of MHC molecules from intracellular endocytic compartments to the DC surface
- down-regulation of antigen internalization
- an increase in the surface expression of co-stimulatory molecules (*e.g.* CD40, CD80, CD86)
- morphological changes and cytoskeleton re-organization
- secretion of cytokines, chemokines, and proteases
- surface expression of adhesion molecules and chemokine receptors.

The presence of stimulatory factors such as co-stimulatory molecules and pro-inflammatory cytokines (*e.g.* IL1, TNF α , IFN α etc) is necessary so that the process of antigen presentation by DCs is stimulatory for T-cells [144].

All the above-mentioned procedures lead to the third step of the cycle. That concerns the priming and activation of effector T-cell responses against cancer-specific antigens. The nature of the immune response is determined at this stage depending on the ratio of effector T-cells versus Tregs. Cytotoxic (CD8+) T-cell activation also requires the presence of IL2 and IL12 that are secreted by CD4+ T-helper cells and DCs, respectively.

In the fourth step, chemokines such as CXCL 9, CXCL10 and CCL5 direct the activated effector CD8+ T-cells to the tumour site.

In the fifth step, the T-cells extravasate through the endothelium after interactions with intercellular adhesion molecule 1 (ICAM-1) and selectins and infiltrate the tumour.

After infiltrating the tumour bed, in the sixth step, they recognize and bind to cancer cells through the interaction between the TCR and tumour antigen bound to MHC class I.

In the seventh step, the above-mentioned interaction between the TCR and tumour antigen bound to MHC class I results in the release of cytokines and cytotoxic granules as well as upregulation of death receptors including

Fas ligand (FasL). The interaction leads to caspase activation and induction of apoptosis and death of the tumour cells with subsequent release of additional tumour antigens and propagation of the cancer-immunity cycle.

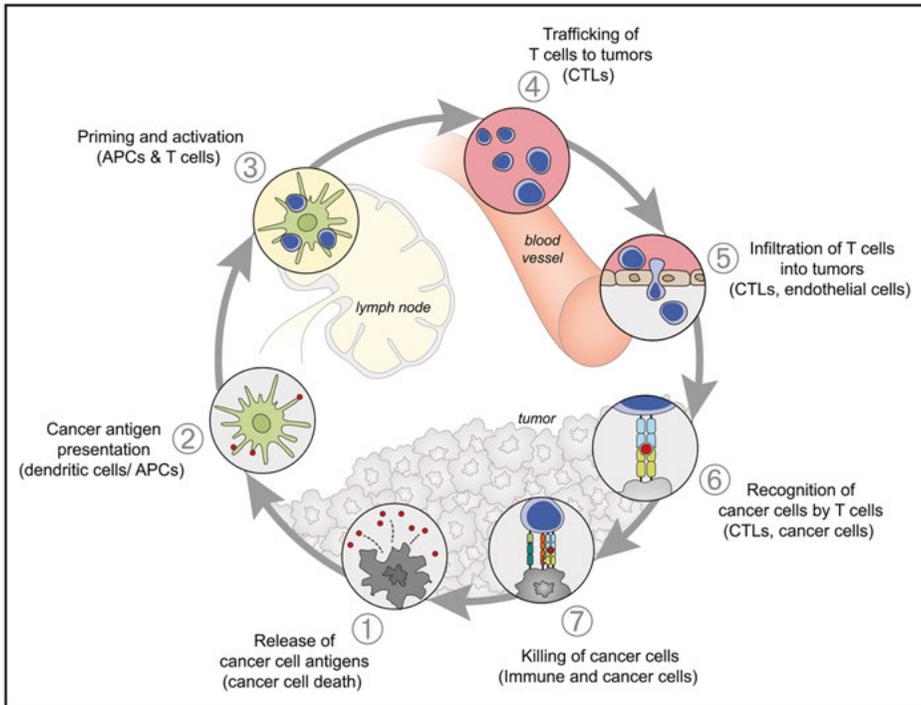


Figure 2. The cycle can be divided into seven major steps and requires the coordination of both immune-stimulatory and inhibitory factors. Stimulatory factors shown in green and inhibitors in red. Abbreviations are as follows: APCs, antigen presenting cells; CTLs, cytotoxic T-lymphocytes. IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; CDN, cyclic dinucleotide; ATP, adenosine triphosphate; HMGB1, high-mobility group protein B1; TLR, Toll-like receptor; HVEM, herpes virus entry mediator; GITR, glucocorticoid-induced TNFR family-related gene; CTLA4, cytotoxic T-lymphocyte antigen-4; PD-L1, programmed death-ligand 1; CXCL/CCL, chemokine motif ligands; LFA1, lymphocyte function-associated antigen-1; ICAM1, intracellular adhesion molecule 1; VEGF, vascular endothelial growth factor; IDO, indoleamine 2,3-dioxygenase; TGF, transforming growth factor; BTLA, B- and T-lymphocyte attenuator; VISTA, V-domain Ig suppressor of T-cell activation; LAG-3, lymphocyte-activation gene 3 protein; MIC, MHC class I polypeptide-related sequence protein; TIM-3, T-cell immunoglobulin domain and mucin domain-3. Although not illustrated, it is important to note that intratumoural T regulatory cells, macrophages, and myeloid-derived suppressor cells are key sources of many of the inhibitory factors. Reprinted from the article *Oncology Meets Immunology: The Cancer-Immunity Cycle* [26] with the kind permission from publisher.

NK-cells also participate in immune surveillance by recognizing and destroying cells that lack MHC class I [145]. However, NK-cell function is not only dependent on missing MHC class I but rather dependent on both inhibitory and activating signals. NK-cells carry inhibitory receptors [*e.g.* killer immunoglobulin like receptors (KIRs), leukocyte inhibitory receptors (LIR)] and activating receptors [*e.g.* Ly49, CD 16, natural killer group 2D (NKG2D)] [146]. The cytotoxicity requires a balance between the signalling systems. KIRs interact with classical MHC class Ia ligands and NKG2D interacts with at least six different ligands with MHC class I homology that are induced by cellular stress such as malignant transformation [147]. NK-cells can be activated and destroy the malignantly transformed cell in which MHC class I is downregulated and NKG2D is expressed [148]. A number of studies have reported that NK-cells in the tumour stroma have an anergic phenotype that is induced by malignant cell-derived transforming growth factor beta (TGF β) [149].

Immunoediting and immune inhibitory mechanisms

In 2001 a study revealed that the immune system controls not only tumour quantity but also tumour quality (=immunogenicity). The basis of the cancer immunoediting hypothesis is that the immune system not only protects the host against development of a tumour but also shapes tumour immunogenicity [30].

The three phases of the immunoediting process are: elimination, equilibrium and escape. In the elimination phase, the innate and adaptive immune systems recognize and destroy cancer cells at an early stage so that no clinical tumour arises. In the equilibrium phase enter those cancer cells that manage to survive the elimination phase. In this phase the adaptive immune system keeps tumour cells in consequential dormancy. However, cancer cells may propagate to the third phase of the immunoediting process, the escape phase, due to a combination of changes in the tumour cells and tumour immunity. This leads to the development of clinically relevant tumours.

Several changes occur in tumour cells leading to immune escape such as loss of tumour antigen expression and increased resistance to cytotoxic effects of the immune system [150]. The above-mentioned changes are caused by genetic instability that is characteristic for tumour cells. The least immunogenic cells escape recognition by the immune system and continue growing through natural selection. Several different mechanisms are operating to suppress tumour specific immunity during the development of a tumour. This leads to the escape phase in which the tumour can continue developing despite of immune surveillance. Many of these suppressive mechanisms are part of the physiologic regulatory system that prevents the immune system from being over-active. Different inhibitory mechanisms may appear during any step of the cancer-immunity cycle, affecting the efficiency of tumour

antigen presentation and activation of cytotoxic T-cells, T-cell tumour infiltration, recognition of tumour cells by T-cells, and cytotoxic killing of tumour cells.

1) Tumour antigen presentation and activation of cytotoxic T-cells

Defective antigen presentation caused by impaired DC function is one of the most important factors of tumour-induced immune suppression. The DC defects observed in cancer are systemic and probably caused by abnormal differentiation of myeloid cells that also leads to accumulation of immature myeloid cells called MDSCs. Factors secreted by tumours such as VEGF, macrophage colony-stimulating factor (M-CSF), IL6, GM-CSF, IL10, TGF β inhibit the differentiation of myeloid progenitors to DCs.

Maturation of DCs is also impaired resulting in development of T-cell tolerance to the tumour [151]. VEGF and IL10 have also been shown to induce PDL1 expression on DCs [152]. PDL1 interaction with its receptor PD1 expressed on activated T-cells leads to inhibition of T-cell activity. CTLA4 is also a negative regulator of T-cell effector function.

2) T-cell tumour infiltration

Chemokines are small, secreted proteins that coordinate homing of immune cells. They direct effector immune cells to the tumour site, however, they alter the TME into a suppressive one by disruption of normal chemokine signalling and attraction of suppressive cell types such as MDSCs and Tregs [153].

T-cell crossing of the endothelium to reach the tumour is impaired. Angiogenic growth factors secreted by the tumour can block the expression of adhesion molecules on endothelial cells thereby inhibiting T-cell extravasation and infiltration. Additionally, endothelial cells can express factors, such as IL10, PDL1, TGF β , that suppress effector T-cells [152].

3) Recognition of tumour cells by T-cells

Tumour cells can fail to express strong tumour antigens or down-regulate their expression of MHC class I thus avoiding recognition by the immune system.

4) Cytotoxic killing of tumour cells

Inhibitory functions can suppress the cytotoxic T-cell function. Those immunosuppressive mechanisms are for example immune suppressive cells, surface molecules expressed on tumour cells and a toxic tumour environment containing soluble mediators that inhibit CTLs such as PDL1: PD1, PDL1: B7.1, TIM-3: phospholipids, BTLA, VISTA, LAG-3, IDO, Arginase, MICA: MICB, B7-H4, TGF β . M2 macrophages, Tregs, MDSCs and hypoxia play a role in this procedure [152].

5) Immunosuppressive cells

T regulatory cells

Tregs are specialized immune cells central that function to maintain self-tolerance and immune homeostasis by suppressing the activation, proliferation, and effector functions of various immune cells [154]. However, Tregs inhibit anti-tumour immune responses in the TME. Increased levels of Tregs have been described in various solid tumour types [155] and in haematological malignancies such as Hodgkin lymphoma [156]. In most solid tumours increased Tregs are associated with a worse prognosis.

Both CD4⁺ and CD8⁺ Tregs exist but the most common and extensively studied are the CD4⁺ Tregs, which are defined as CD4⁺CD25⁺FOXP3⁺. The nuclear transcription factor Fork-head box P3 (FoxP3) is not only a marker for Tregs but also has important function in the development of the regulatory phenotype [157].

Historically, Tregs were classified in two subsets: natural, nTregs, that are derived in the thymus and can suppress cells of both innate and adaptive immunity or induced, iTregs, that are derived from naïve T-cells in the periphery under suppressive conditions [154]. Thymically derived CD4⁺CD25⁺Foxp3⁺ Tregs are a relatively homogeneous population until they migrate out into the periphery. A subpopulation of these cells can develop phenotypic characteristics similar to conventional memory and effector T-cells in the periphery. This phenotypic change enables their subsequent migration to lymphoid and non-lymphoid tissues to maintain immune homeostasis. In the periphery, Tregs may develop from conventional T-cells (*i.e.*, those that exited the thymus as CD4⁺CD25⁻Foxp3⁻) [157, 158].

Additionally, Treg subsets can be defined based on the expression of chemokine receptors and adhesion molecules. There is increasing evidence that Tregs mediate their suppressive function through a variety of different mechanisms, suggesting that there is functional specialization depending on the type of immune response and where it is localized. One mechanism involves the secretion of IL10, which serves to directly or indirectly inhibit effector T-cell responses. Tregs also secrete IL35 and TGF β to induce conventional CD4⁺ T-cells to differentiate into Tregs, thereby skewing the ratio of Tregs to T-helper cells during an immune response. Equally as important, cell surface molecules such as CTLA4 also participate in Treg cell-mediated suppression. CTLA4 inhibits DC-mediated T-cell stimulation by binding to CD80 and CD86, which leads to downregulation of these co-stimulatory molecules on the DC and induction of IDO, an enzyme that depletes tryptophan from the microenvironment. Tregs also have the ability to directly destroy effector T-cells through release of cytolytic mediators or through induction of apoptotic pathways in target effector cells. Thus, understanding the mechanisms by which Tregs exert their suppressive function has broad implications for drug development strategies aimed at treating cancer [91, 154,

159]. In murine models it has been shown that one part of the effector mechanisms of anti-CTLA4 therapy is Treg depletion via ADCC. The dense expression of CTLA4 on murine Tregs in the tumour mediates selective killing of the same [160].

Myeloid-derived suppressor cells

Myeloid derived suppressor cells are a heterogeneous population of cells that is defined by its myeloid origin, immature state and ability to suppress T-cell responses. MDSCs regulate immune responses and tissue repair in healthy individuals. Their population expands during pathological conditions such as inflammation, infection, trauma and cancer due to induction of a block in the differentiation of various immature myeloid cells [151].

Studies have shown expansion of MDSCs in patients with various solid tumours [161]. The impact of MDSCs in cancer could be described as a two-staged effect [162, 163]:

- The first stage is abnormal myelopoiesis and recruitment of MDSCs into the tumour tissue.
- The second stage is the active production of MDSC-derived cytokines and cell–cell interactions within the microenvironment resulting to progression of cancer.

MDSCs limit T-cell responses and their infiltration into the TME, thus promoting tumour immune escape [164]. Whether MDSCs mediate antigen-specific or nonspecific suppression of T-cell responses in the TME remains still unanswered.

In mice, MDSCs are defined by the characteristic co-expression of myeloid lineage differentiation antigens Gr-1 (also known as Ly6C/G) and CD11b (α M-integrin). MDSCs with a monocytic morphology have a CD11b+Ly6G⁻Ly6C^{high} phenotype, whereas granulocytic MDSCs have a CD11b+Ly6G+Ly6C^{low} phenotype. The exact roles of both MDSC subpopulations in pathological conditions are not fully understood yet [164].

In humans, MDSCs are defined as the CD14⁻CD11b+CD33+CD15+ phenotype or cells that express the CD33 marker but lack the expression of markers of mature myeloid and lymphoid cells and the MHC class II molecule. The identification and isolation of human MDSCs subsets have been difficult because of the heterogeneous characteristics of these immature cells. Data suggest a significant diversity in the MDSCs subsets in different human cancers [151].

The expansion and activation of MDSCs is caused by various factors produced by tumour cells, stroma cells associated with tumours and activated T-cells. MDSCs expansion is achieved by stimulation of myelopoiesis and by triggering a cascade through the signalling of Janus tyrosine kinase (JTK) and signal transducer and activator of transcription 3 (STAT3). Factors such

as cyclooxygenase (COX) 2, prostaglandins, stem cell factor (SCF), M-CSF, GM-CSF, IL6 and VEGF can trigger the STAT3 signalling.

Upregulation of nuclear factor (NF)-kappa B is triggered by activation of MDSCs through STAT1 and STAT6 signalling. IFN γ , IL4, IL13, TLRs, and TGF β can activate these signalling pathways [151].

Activation of NF-kappa B leads to MDSC suppressive activity through upregulation of two enzymes, Arginase-1 and inducible nitric oxide synthase (iNOS). These enzymes metabolize the amino acid L-arginine to produce urea and L-ornithine and NO, respectively and they suppress T-cells through different mechanisms [165, 166]. Activation of NF-kappa B induces production of suppressive cytokines (*e.g.* TGF β) which in turn promotes the induction of Tregs [167].

MDCs can also induce antigen specific CD8+ T-cell tolerance that is another mechanism for tumour-immune escape [168]. Other mechanisms for immune suppression by MDSCs have also been proposed, such as down-regulation of L-selectin on T-cells and depletion of cysteine from T-cells both inhibiting cell activation [169]. Understanding the development, activation and effects of MDSCs is crucial for effective therapeutic strategies.

Specifically, in patients with advanced melanoma, monocytic MDSCs in the blood are the main cause of monocyte-associated immune suppression. Furthermore, they negatively affect patient survival and correlate inversely with the number of functional antigen-specific T-cells [170].

Of interest is the selective killing of suppressive immune cells from certain types of cytostatic drugs at specific doses. See below for more information.

CD40 ligand and AdCD40L

CD40 plays a profound role in the stimulation of the adaptive immune response. Due to that, clinical studies with CD40-mediated cancer treatment have been performed. The co-stimulatory receptor CD40 is a type I transmembrane protein and it is expressed on B-cells, DCs, monocytes, platelets and macrophages as well as by non-hematopoietic cells such as epithelial, endothelial cells and fibroblasts [171].

The ligand of CD40 (CD154, CD40L) is a type II transmembrane protein and a member of the TNF superfamily. Soluble CD40L expresses activities similar to the transmembrane form but is in general not as strong since the trimerised structure seen in the membrane is resolved and soluble CD40L is hence presented as a monomer. CD40L is an important endogenous danger signal with numerous functions. These functions depend on which cell expresses CD40 receptor and which cell produces CD40L. The biological responses range from immune cell activation to tumour cell apoptosis and effects on the tumour vasculature. Activated T- and B-cells as well as plate-

lets express CD40L. Under inflammatory conditions CD40L is induced on NK-cells, mast cells *etc* [172].

Interactions between CD40 and CD40L exert profound effects on DCs, B-cells, and endothelial cells, as well as many other cells of the hematopoietic and non-hematopoietic components. The CD40 signalling on tumour cells may lead to growth arrest or apoptosis improving the outcome of the treatment. In contrast, normal cells do not go in apoptosis under CD40 signalling, thus therapies based on stimulation of CD40/CD40L interaction could represent interesting therapeutic approaches [172].

Specifically, engagement of CD40 by multimeric CD40L causes redistribution of CD40 to membrane lipid rafts and a conformational change that recruits adapter molecules known as TNF receptor (TNFR) - associated factors (TRAF) to at least two distinct binding sites on the CD40 cytoplasmic tail [173, 174]. TRAFs then recruit TRAF-interacting kinases. Together they influence a number of signal transduction pathways, such as the nuclear factor- κ B, MAPK and c-Jun-NH2-kinase (JNK) pathways [175]. Target genes of CD40 signalling regulate apoptosis, cell cycle progression, cytokine production, expression of cell surface immune-modulators and TNF family members and other pathways. Second extracellular signals co-operate with the CD40 signalling pathway, inducing overlapping responses or triggering others.

AdCD40L is an adenoviral-based immunostimulatory gene therapy. The adenoviral backbone stimulates TLRs while CD40L potentiates the Th1 type of immunity. AdCD40L can be used for both *ex vivo* and *in vivo* gene transfer. For example, it can be used for *ex vivo* gene modification of tumour cell vaccines that are then used for *in vivo* delivery but can also be used for direct *in vivo* administration by intratumoural injections. Intratumoural injection of AdCD40L induces CD40L expression at the tumour site of solid tumours [176]. DCs present at the tumour site are already loaded with tumour antigens. DC maturation is induced by CD40L gene expression by cells at the injection site and leads to activation of anti-tumoural T-cell responses (Figure 3).

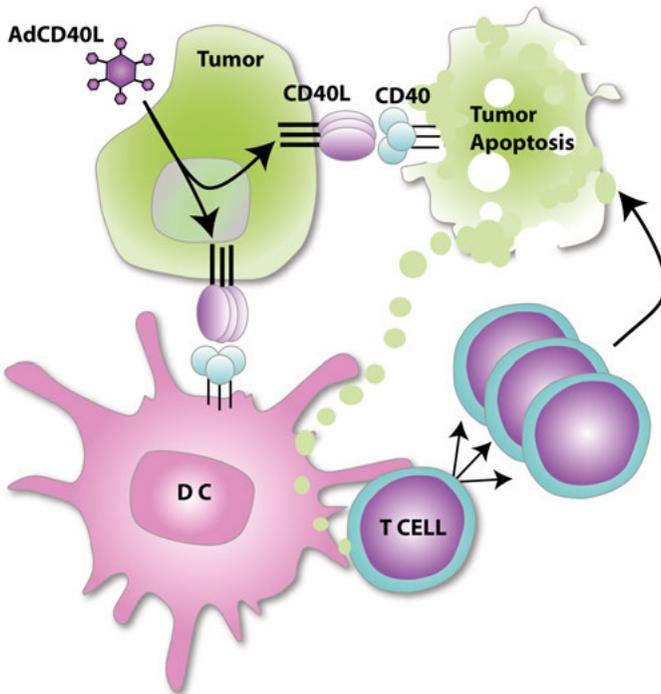


Figure 3. AdCD40L, an adenoviral vector carrying the human CD40L gene, infects tumour cells, DCs, or other cells at the injection site, upon intratumoural injection. AdCD40L delivers the CD40L gene into the cells whereupon the virus is destroyed. This gene will be transcribed and translated to CD40L protein via the tumour cell translation system. The CD40L protein is then transported to the tumour cell surface where it will trimerise upon binding to CD40 on adjacent cells. CD40L will then interact with CD40 receptors present on the surrounding immune cells, leading thus, to immune cell activation. CD40L is one of the most potent stimulators of the immune system. CD40⁺ tumour cells will undergo apoptosis upon CD40L ligation. DCs will become efficient antigen presenters to T-cells and other lymphocytes. Apoptotic tumour cells will be taken up by the mature DCs and processed in order to activate a broad anti-tumour T-cell response.

Immunostimulatory gene therapy with AdCD40L has shown efficacy in various murine models, and safety studies have been performed on dog patients with MM and in various human clinical trials. The leukemic cells can easily be collected from patients with B-cell leukaemia for in vitro transfer. Thus, the first clinical studies, where the AdCD40L was tested were performed in this group of patients. A CD40L-expressing tumour cell vaccine was prepared by ex vivo transduction of tumour cells and/or fibroblasts to treat patients with B-cell malignancy. Bladder cancer was the first solid tumour in a human trial using AdCD40L for direct gene delivery. It was a clinical phase I/II trial and 8 patients were included [177]. Five of them had invasive high-grade tumour and were scheduled for cystectomy, and the remaining three had superficial Ta tumours. All patients received

three intra-bladder treatments, one week apart. The first three patients in the invasive tumour cohort received low dose AdCD40L (1×10^{11} VP), and the rest of the patients received high dose (1×10^{12} VP). There were no dose-limiting toxicities or adverse events connected to the vector. Of the five patients with invasive tumours, three patients did not have remaining high-grade tumour cells. One of them had no high-grade tumour in the bladder, however, a remaining metastasis in the ureter and the last did not show any response. The three patients with superficial tumours still had tumour cells left post-therapy, although the size was reduced in one of them. The effector marker IFN γ was significantly increased in bladder biopsies post-treatment [177].

In a pilot study of local AdCD40L treatment, 19 cases of canine melanoma (14 oral, four cutaneous, and one conjunctival) were included. One to 6 intratumoural injections of AdCD40L were given every 7 days, followed by cytoreductive surgery in 9 cases. Tumour tissue was infiltrated with T- and B-lymphocytes after treatment, suggesting immune stimulation. Median survival was 160 days. Five complete responses, eight partial responses, four stable and two PD statuses according to the World Health Organization response criteria were observed [178].

Conditioning

Due to the immunosuppressive environment in tumours, the effect of immunotherapies is hampered. However, if immunosuppressive cells are reduced prior treatment, the effect is expected to be enhanced. One way of reducing immunosuppression is to treat the patients with chemotherapy as a conditioning prior immunotherapy.

For almost two decades ago, clinical trial patients with metastatic MM received conditioning with two different cytotoxic agents: fludarabine and cyclophosphamide. This strategy resulted in a 50% response rate and a long-term persistence of adoptively transferred T-cells. Evidence indicate that lymphodepletion -with fludarabine and cyclophosphamide- prior to adoptive transfer of tumour-specific T-lymphocytes enhances the efficacy of the treatment by eliminating Tregs and competing elements of the immune system [179].

Cyclophosphamide is a chemotherapeutic that belongs to the group of alkylating agents. The cyclophosphamide is efficient against various cancers such as small cell lung cancer, breast cancer, ovarian cancer and myeloma. However, it has no cytotoxic effect against MM.

Ghiringhelli et al demonstrated in their animal experiment that depletion of Tregs after a single injection of cyclophosphamide leads to potentiation of tumour-specific immunotherapy [180]. Thus, it has the capacity to reduce the number of immunosuppressive Tregs, allowing a new anti-tumour immune response to thrive. Low dose of cyclophosphamide has the capacity to

stimulate NK-cells. Conditioning by cyclophosphamide in combination with T-cell therapy or oncolytic adenoviruses is widely used in clinical trials in order to enhance the efficacy of immunotherapy [181].

Aims of the Doctoral Project

The overall aim of this doctoral project is to improve the treatment of patients with MM by immunostimulating gene therapy (AdCD40L) and by BRAF inhibition in rare subtypes of melanoma.

I) We have designed and carried out a translational phase I/IIa trial for patients with advanced disease using intratumoural injections of AdCD40L.

The specific objectives were:

- To evaluate the toxicity and anti-tumoural effects (both local and systemic) of local AdCD40L immunostimulatory gene therapy (**paper I**).
- To examine the toxicity and anti-tumoural effects of combination therapy with low dose cyclophosphamide and AdCD40L (**paper I**).
- To analyse plasma protein markers at baseline and post-AdCD40L therapy in order to identify potential biomarkers in plasma which might be of biologic, prognostic or therapeutic significance (**paper II**).
- To evaluate the monitoring of the radiological effects of the AdCD40L therapy with or without cyclophosphamide \pm radiotherapy in patients with disseminated MM (**papers I and III**).
- To investigate the potential of DW-MRI and FDG-PET/CT for early prediction of AdCD40 treatment response in patients with metastatic MM in terms of OS (**paper III**).

II) We used the BRAF-inhibitor vemurafenib as a treatment in single patient cases:

- The aim was to investigate whether vemurafenib could safely be used in order to prolong gestation in a pregnant woman with BRAF-mutated metastatic MM. We reported the first case –being still the only report of administration of vemurafenib during pregnancy (**paper IV**).
- The aim was to investigate whether vemurafenib would be beneficial in a patient with BRAF-mutated metastatic ocular melanoma. We reported the first case of a patient with metastatic ocular melanoma who benefited from treatment with the BRAF inhibitor vemurafenib (**paper V**).

Subjects and Methods

AdCD40L trial

The AdCD40L trial is presented in Paper I-III.

Study scheme

In our open-label phase I/IIa trial 24 patients with metastatic MM were enrolled. The trial consisted of three parts:

- In the first part (cohort 1) six patients received four weekly ultrasound-guided intratumoural injections of AdCD40L.
- In the second part (cohort 2) nine patients received low-dose intravenous cyclophosphamide conditioning (300 mg m^2) 1–2 days before the first and fourth injection of AdCD40L.
- In the third part (cohort 3) nine patients received the same treatment as in cohort 2 with an additional single-fraction (8 Gray) to the metastasis intended to be injected. The fraction was given one week prior to the first intratumoural injection.

The patients were monitored for a period of 10 weeks in which they were sampled for blood chemistry, haematology and immunology evaluation at multiple time points. All patients underwent pre-treatment whole-body (WB) DW-MRI and FDG-PET/CT at 2 as well as at 6 weeks after the last AdCD40L injection. Morphological tumour response was assessed by MRI and CT according to RECIST 1.1 criteria while functional/metabolic response by DW-MRI and FDG-PET/CT.

AdCD40L medicinal product

AdCD40L is an adenoviral serotype 5, group C, vector deleted of E1 and E3, carrying the transgene for human CD40L driven by a RSV promoter [182]. The vector was cloned at the Center for Cell and Gene Therapy (CAGT) at Baylor College of Medicine, Houston, TX. AdCD40L vector diluted in 500 μL Ringer lactate solution to a final dilution of 2.5×10^{11} VP. The adenovirus was kept at $+4^\circ\text{C}$ until injection.

Paper I and II

In paper I and II, data from cohorts I and II are presented.

Immunological analyses

Biopsies and plasma

Protein lysates from the frozen biopsies were obtained with a standard RIPA cell lysis buffer. Lysates as well as plasma samples taken at baseline (pre) and at 3 weeks post-treatment initiation (post), were analyzed using ProSeek Multiplex Inflammation^{96×96} (Olink Biosciences AB, Uppsala, Sweden).

Proseek multiplex is an antibody-based method for multiplex protein detection. They used the technique of proximity extension assay. The values are reported in relative units -referred to as normalized protein expression (NPX)- since the data generated cannot be converted to absolute concentrations.

Adenovirus IgG ELISA was used to measure antibodies against adenovirus (GenWay, San Diego, CA, USA). Immunohistochemistry using aCD40 and aCD3 was performed by the routine diagnostics facility at the Department of Pathology and Cytology, Uppsala University Hospital.

Peripheral blood mononuclear cells

Flow cytometry was used for the analysis of peripheral blood mononuclear cells (PBMCs) at baseline, at week 5 and at week 9 after treatment initiation. The PBMCs were thawed, washed with phosphate-buffered saline (PBS) and incubated at room temperature for 10 min with FcR blocking reagent (Miltenyi Biotech, Germany). Thereafter, the cells were incubated with antibodies targeting CD3, CD4 and CD127 for 30 min at 4 °C followed by fixation and permeabilisation. The cells were then incubated with an antibody targeting FoxP3 for 30 min at 4 °C. Finally, after a final wash with PBS with 0.5% bovine serum albumin (BSA) the cells were resuspended in PBS-BSA.

Cells were analysed in a BD FACS Canto II (BD Biosciences, San Jose, CA, USA) and data was evaluated in Flow Jo (Tree Star, Ashland, OR, USA). Before gating of specific cell subsets, dead cells and duplets were removed by gating.

T-cells were determined as CD3 positive cells - either of effector type (CD4⁻, CD127⁺), regulatory type (CD4⁺FoxP3⁺CD127⁻) or T-helper cells (CD4⁺, CD127⁺). NK-cells were defined as CD3⁻ negative cells expressing CD16 and CD56. CD14⁺CD11b⁺CD33⁺ cells that lacked HLA-DR were classified as monocytic myeloid suppressors while CD11b⁺CD33⁺ cells lacking HLA-DR and CD14 as granulocytic myeloid suppressors.

Statistical analyses

The following statistical methods were used:

- Kaplan-Meier method and log-rank test in order to study the difference in 6-month survival between the two cohorts.
- Correlation analyses were performed using the nonparametric Spearman's correlation analysis.
- Student's *t*-test with Welsh (unpaired) or Wilcoxon (paired) correction to study the levels of caspase 8, CD40, IP10, CXCL9, TGF β and IL8 pre- and post-treatment (in paper I).
- Wilcoxon matched pairs signed rank test: to determine differences between pre and post-treatment samples. Mann–Whitney U testing was used or comparisons between unpaired groups (in paper II).

Paper III

In paper III data from cohorts 1, 2 and 3 are included.

Radiological parameters

In order to verify our hypothesis we selected two different lesions in each subject: the injected metastasis and one more lesion that had both high SUVmax but also was measurable on MRI according to RECIST 1.1. ROIs were drawn over the same selected lesions in both FDG-PET and DW-MRI images to measure their SUVmax as well as to extract their ADC, D and f values. Fold changes in SUVmax (Δ SUVmax), ADC (Δ ADC), D (Δ D), f (Δ f), as well as of the lesion size were statistically assessed. Δ -value ≥ 1 indicates increase, respectively Δ -value < 1 decrease of the chosen parameter.

For the assessment of the morphological changes in the tumours the RECIST 1.1 criteria were used. Tumour metabolic activity was assessed according to EORTC criteria: progressive metabolic disease (PMD) was defined as $\geq 25\%$ increase while partial metabolic response (PMR) was defined as $\geq 15\%$ decrease of mean SUVmax in lesions. Neither PMR nor PMD was considered as stable metabolic disease (SMD) [183]. Until now, no criteria exist regarding the acquisition of ADC, D and f values in order to assess treatment response and obviously neither for assessment of early response to immunotherapy.

Statistical analyses

The analyses were first conducted with inclusion of all patients who underwent at least one radiologic examination post-AdCD40L therapy (n=21) and then by excluding these patients who did not undergo all examinations (n=13).

The following statistical methods were used:

- Kaplan-Meier method and log-rank test in order to compare the OS in patients with ΔADC , ΔD , Δf , ΔSUV_{max} and fold change of lesion size ≥ 1 and patients with values < 1 .
- Correlation analyses were performed using the nonparametric Spearman's correlation analysis.
- One-way ANOVA followed by planned contrasts was performed when data were normally distributed in order to investigate differences between the three cohorts, RECIST 1.1 and EORTC criteria regarding ΔADC , ΔD , Δf , ΔSUV_{max} and OS.
- Kruskal-Wallis followed by Mann Whitney *U*-tests were performed when data were not normally distributed. Direct comparisons between the SUV_{max} pretreatment, at week 5 and week 9 performed with the use of paired t-tests.

Vemurafenib case reports

Two rare cases, one of a pregnant patient with cutaneous BRAF-mutated MM and one of a patient with BRAF-mutated metastatic ocular melanoma were treated with vemurafenib. The conduct of clinical trials is restricted due to the extremely low prevalence of the above conditions therefore single patient cases were studied.

Paper IV

Review and collection of patient's data in the electronic patient annotation system. Blood samples from the mother, the newborn and the umbilical cord were collected after the birth and were tested for the presence of vemurafenib.

Paper V

Review and collection of patient's data in the electronic patient annotation system. Every report including the pathology report of the primary tumour was reviewed.

Results and Discussion

Paper I

1) Safety of AdCD40L treatments

The treatment with AdCD40L ± cyclophosphamide was well tolerated. Four patients in cohort 1 and eight in cohort 2 experienced transient grade I–II adverse events. Only one patient (#12) experienced grade III symptoms; pain related to the biopsy and anaemia. However, the latter could represent anaemia of chronic disease. None of the patients experienced grade IV adverse events. Vitiligo, flu-like symptoms, fever and fatigue were regarded to be directly connected to the virus, whereas pain at the injection site, nausea and vomiting in relation to treatment were considered to be due to the injection procedure. The most common adverse events connected to AdCD40L were transient fever, pain at the injection site, transient increase of liver enzymes, mild nausea. The frequency of adverse events connected to the virus was higher in cohort 2, indicating the higher likelihood for the virus to induce immune activation in patients conditioned with cyclophosphamide.

2) Clinical results according to FDG-PET/CT and WB-MRI scans

AdCD40L therapy ± low dose cyclophosphamide did not reduce tumour load according to RECIST 1.1. Nevertheless, it is known that survival advantages from immunotherapy are not regularly accompanied with ORs [49]. In several patients we observed lower metabolic tumour activity post-treatment in PET/CT scans:

Cohort 1: Two patients had reduced tumour activity in the injected metastasis and in a few other distant metastases at the first post-treatment PET/CT examination.

Cohort 2: Four patients experienced reduced metabolic activity in the injected metastasis. Two patients (#7, #8) showed reduced SUVmax in all metastases and one patient had mixed response in both post-treatment PET/CT examinations. Two patients experienced increased metabolic activity in a few metastases in the first post-treatment PET/CT scan but stable metabolic activity in the second one. Two patients had an increase in tumour activity in both post-treatment PET/CT scans.

3) Overall survival of AdCD40L-treated patients

The median survival in cohort 1 was 18 weeks and in cohort 2 was 34 weeks. At 6 months, one of six patients was alive in cohort 1 and seven of nine were still alive in cohort 2. The difference between the groups was significant. However, the OS was similar in both groups.

4) Cyclophosphamide did not significantly affect Treg or effector T-cell levels

The Treg levels were stable in the patients in both cohorts except in patient #14 who experienced a significant decrease. The activated CD4+ T-cell levels were stable in cohort 1. In cohort 2, the levels were significantly higher and the three patients that benefitted most from the treatment (#7, #8, #14) had the highest levels. The activated CD8+ T-cells remained stable during therapy and were not affected by conditioning. The longest surviving patients (#7, #8 and #14) had higher levels than the other patients with the exception of patient #13. The levels of infiltrating CD3+ T-cells in the tumour biopsies were higher post-treatment in cohort 2 compared to cohort 1.

5) CD40 levels correlate to caspase 8

Caspase 8 was significantly increased in biopsies post-treatment indicating cell death, however no correlation to OS was observed. Cell death induced by AdCD40L can be due to tumour killing by the direct interaction with CD40+ tumour cells. A high level of intratumoural CD40 correlated significantly to a high level of caspase 8.

6) Decreasing levels of IL8 post-treatment correlates to overall survival

The fold decrease of intratumoural IL8 pre *versus* post-therapy correlated significantly to longer OS.

All the patients were heavily pre-treated, resulting in a poor immunological capacity. The patients experiencing the longest survival had significantly lower Treg levels and significantly higher levels of CD4+ and CD8+ effector T-cells as compared to the other patients. Malignant melanoma expresses CD40, making it a suitable target for CD40L-mediated apoptosis [172]. In addition to its immunostimulatory effect, we have shown that CD40L might have induced a signalling cascade leading to apoptosis of CD40+ tumour cells.

Treatment with intratumoural AdCD40L was shown to be less resource demanding than T-cell therapy and well tolerated. Local as well as distant decreased uptakes were seen in PET/CT scans in seven patients. The results are encouraging and further studies are warranted.

Paper II

1) AdCD40L induces effector T-cell responses

In our material, all patients had an increased Teff/Treg ratio post-treatment. Previous human studies showed that local AdCD40L administration decreases the number of Tregs while simultaneously stimulates Th1-type of immunity. Tregs hamper anti-tumour immune response by suppressing T-effector cells. We demonstrated a shift in the Teff/Treg ratio in favour of Teff suggesting efficacy of the AdCD40L therapy. However, this increase in the ratio did not lead to higher levels of IFN γ . A possible explanation is that since IFN γ is acting in a cell-to-cell contact manner it is quite difficult to detect in the patients' blood.

Moreover, TNFR1 and TRAILR2, death receptors used by T-cells to induce tumour cell apoptosis, were increased post-therapy. The majority of the patients with the longest OS had higher levels of TNFR1 and TRAILR2 both pre- and post-treatment. TNF exerts its functions even via TNFR2; however, TNFR2 is related not to apoptosis but to T-cell survival. In our two first cohorts, TNFR2 was found to be increased post-AdCD40L therapy. Almost all the patients with increased post-therapy levels of Tregs also had increased TNFR2-levels post-treatment.

In conclusion, AdCD40L induced T-cell activation, which might be of clinical importance.

2) Granulocytic MDSCs correlate with poor survival

In the patients with the shortest survival an increase of the level of gMDSCs post-therapy was observed. However, no correlation was found between the number of MDSCs and OS. Furthermore, we did not observe any correlation between other suppressive cells as well as molecules and the level of MDSCs.

3) Proteins were altered post-treatment

Proteins connected to immunosuppression

In our material, both pre- and post-values of the key immunosuppressive cytokine IL10 correlated inversely to OS. IL10 has various functions including impairment of Teffs' migrations capacity and in targeting DCs [184]. The majority of patients with a survival shorter than 6 months had high levels of different immunosuppressive proteins apart from IL10 *i.e.* TGF β 1, PlGF, IL8 and Flt3L.

There is evidence that Flt3L stimulates the expansion as well as the function of MDSCs by activating the transcription factor STAT3 [185, 186]. Our data was indeed in line with the above-mentioned observation since the post-therapy level of Flt3L in plasma was higher among patients with shorter survival and in five of these patients the level of mMDSCs were higher.

We showed, in paper I that the decrease of the pro-inflammatory chemokine IL8 intratumorally correlated to OS. Plasma proteomic confirmed this observation by showing that the group of patients with short survival had very high post-therapy plasma levels of IL8 while the responding patients had lower levels.

Proteins connected to immunity

Selectins are adhesion molecules and their role is to initiate leukocyte rolling in order to assist the recruitment of leukocytes to the inflammation site. E-selectin is expressed on activated endothelium. E-selectin dependent attachment of T-cells to the endothelium is induced upon CD40/CD40L ligation [187]. In our material the level of vascular E-selectin was significantly raised post-therapy. Moreover, the plasma level of E-selectin was higher in patients with the longest survival. CD40/CD40L interaction induced by AdCD40L-treatment may have led to increased levels of E-selectin that in turn led to higher recruitment of leukocytes to the sites of inflammations and thereby improved immunity.

The post-value of CD6, a lymphocyte surface receptor that promotes the adhesion between APCs and T-cells [188], correlated to longer OS.

The level of another protein, SCF, was higher both pre- and post-therapy in patients with the longest survival. SCF is known to facilitate the recovery of DCs [189].

These findings strengthen our hypothesis that therapy with AdCD40L can induce positive immune responses that correlate to prolonged survival of the patients.

The results of paper II suggest that CD40 stimulation has a place in the treatment of patients with metastatic melanoma, however improvements of the treatment scheme are warranted.

Paper III

1) ΔADC and $\Delta D \geq 1$ of the injected metastasis were associated with better OS

All evaluable patients (n=21)

The median OS of the patients with $\Delta D \geq 1$ (black line) compared to the median OS of the patients with $\Delta D < 1$ (grey line) at both week 5 and week 9 was significantly better as it is illustratively shown in the figure below.

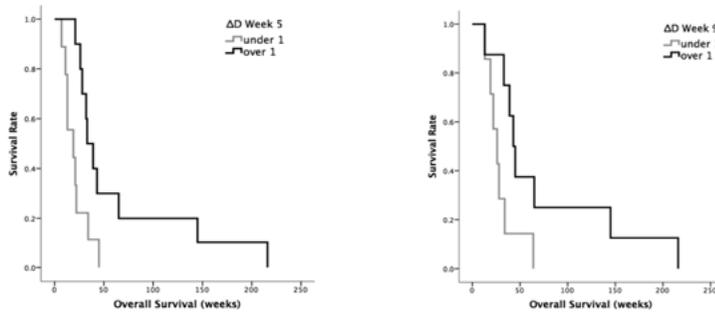


Figure 4: Graphs show the overall survival of patients with metastatic melanoma treated with AdCD40L gene therapy assessed by ΔADC , ΔD and ΔSUV_{max} of the injected metastases.

Patients who underwent all examinations (n=13)

For patients with $\Delta D \geq 1$ compared with those with $\Delta D < 1$ at week 5 a similar trend in OS difference was observed. The median OS of the patients with $\Delta D \geq 1$ compared with the median OS of the patients with $\Delta D < 1$ at week 9 was significantly better. The median OS of the patients with $\Delta ADC \geq 1$ was significantly better compared to the median OS of the patients with $\Delta ADC < 1$ at week 9.

These data imply that the increase of D value in the injected metastases from baseline to post-therapy DW-MRI at both week 5 and week 9 could predict longer OS. For the ADC value, the same pattern was observed at week 9 in the patients with better general condition who underwent all imaging examinations.

2) Increase of ADC and D in the injected metastases correlated to OS

The increase of ADC and mainly D in the injected metastases correlated significantly to OS but changes of the SUV_{max} at these points did not.

It is unclear if the increases in D/ADC indeed reflect treatment response. Theoretically, a rapid progression of the tumour can lead to necrosis resulting in an ADC-increase. Hence, the positive correlations between D/ADC and OS have to be interpreted with caution since it can partly be explained by differences in tumour aggressiveness as well as due to the low number of patients. However, we identified five patients for whom FDG-PET/CT resulted in PMR and also had an increase in D/ADC. This finding indicates that not all D/ADC increases can be explained by tumour progression.

One plausible explanation for that the changes of the SUV_{max} did not correlate to OS is a possible inflammatory reaction in the metastases that counteracted the treatment-induced response. An inflammatory response could increase the cellularity in metastases resulting in a lower D value. However, one could argue that this type of increased cellularity could be

counterbalanced both by an inflammatory induced oedema as well as by a deterioration of the function of the cell membranes and apoptosis due to our treatment. This could lead to increased D as a result of the increased movement of the water molecules in the increased extracellular space.

The majority of the patients had a fold change of the size of the injected metastasis > 1 and it was negatively correlated to OS both at week 5 and at week 9. An increase with $\geq 30\%$ of the size of the injected lesion at week 9 correlated indeed to shorter OS.

3) Treatment response assessment

Table 1: *Patients evaluated according to RECIST 1.1*

	N=21		N=13	
	SD	PD	SD	PD
Post I (week 5)	11	10	9	4
Post II (week 9)	8	8	8	5

Table 2: *Patients evaluated according to EORTC*

	N=21			N=13		
	SMD	PMR	PMD	SMD	PMR	PMD
Post I	11	6	4	7	3	3
Post II	8	5	3	7	4	2

4) Correlation between FDG-PET/CT and DW-MRI in the injected lesion

Both ΔADC and ΔD values were significantly negatively correlated to ΔSUV_{max} at the first post-therapy radiological evaluation. This negative correlation of ΔADC and ΔD with ΔSUV_{max} indicates that as ADC and D values were increased, the tumour viability was decreased. Δf values correlated to ΔSUV_{max} at week 9. This correlation suggests that when the metabolic activity of the tumours increased, the f had also increased. It may reflect the existence of a more organized capillary network that supports the tumour.

Figure 5 illustrates the radiographic images of a 69-year-old woman with disseminated cutaneous MM (cohort 3). Four intratumoural injections with AdCD40L were given to a lymph node metastasis in fossa supraclavicularis. The patient had SMD post-therapy according to EORTC criteria while an increase in ADC/D in the injected metastasis was observed at both DW-MRI scans post-therapy. The size of the metastasis increased with more than 30%. Hence, the case was assessed as PD according to RECIST 1.1. A decrease of the $f\%$ value was observed.

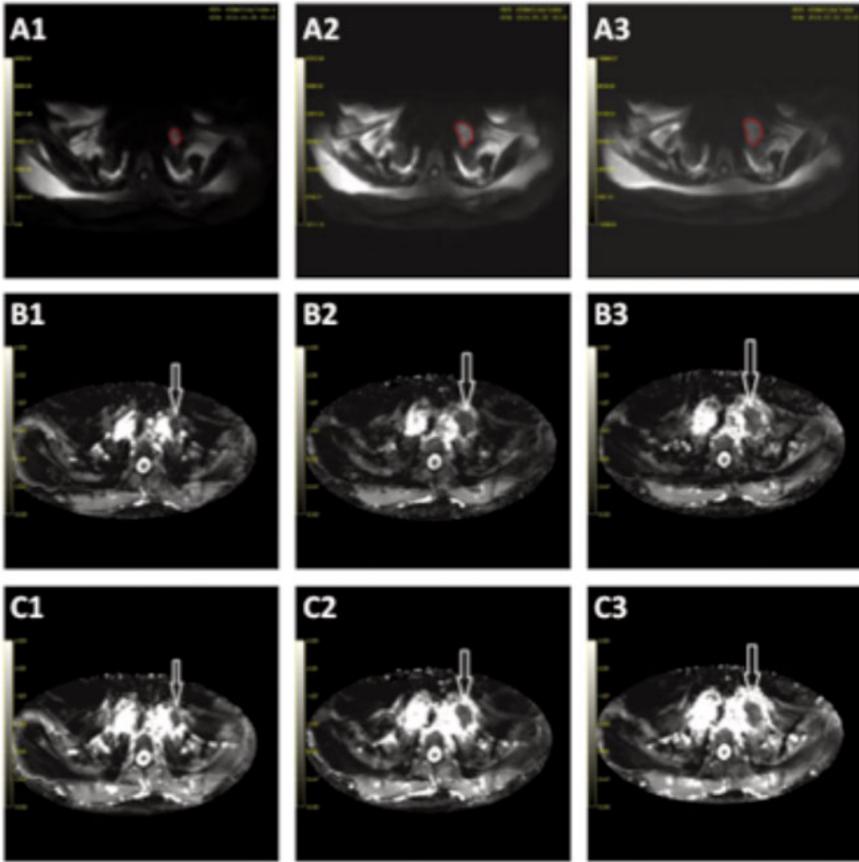


Figure 5.

A1-A3: Diffusion-weighted MR image (DWI) at $b = 900 \text{ s/mm}^2$ on the axial plane. The red circles indicate the injected metastasis at baseline (A1), at week 5 (A2) and at week 9 (A3).

B1-B3: D values were measured $0.49 \text{ mm}^2/\text{s}$ at baseline (B1), $0.78 \text{ mm}^2/\text{s}$ at week 5 (B2) and $1.03 \text{ mm}^2/\text{s}$ at week 9 (B3). The arrows indicate the injected metastasis.

C1-C3: ADC values were measured $0.95 \times 10^{-3} \text{ mm}^2/\text{s}$ at baseline (C1), $1.06 \times 10^{-3} \text{ mm}^2/\text{s}$ at week 5 (C2) and $1.18 \times 10^{-3} \text{ mm}^2/\text{s}$ at week 9 (C3). The arrows indicate the injected metastasis.

The $f\%$ values were measured 30.4, 22.2 and 14.6 respectively during the timepoints mentioned above. The SUV_{max} of the lesion was 16.2, 18.4, 17.8 respectively. The size of the lesion was 30mm, 35mm, 41 mm respectively.

One of the most important oncological challenges is to optimize treatment for every patient *i.e.* giving the most effective drug/drugs while at the same time avoiding severe toxicity [190]. Although it is known since a decade ago that treatment with immunotherapeutic agents may not lead to shrinkage of the tumours, in the majority of clinical trials with such agents, the RECIST 1.1 criteria for CT/MRI are still used for assessing treatment benefit [118].

To our knowledge, this is the first report on DW-MRI for evaluation of tumour responses in MM patients treated with immunomodulation. Our results suggest that DW-MRI is superior to FDG-PET/CT for early prediction of tumour response, in terms of OS. DW-MRI could represent a useful method for early evaluation of treatment response to immunotherapy; however, studies evaluating DW-MRI in larger patient cohorts are warranted.

Paper IV

Case presentation

In paper IV we presented the case of a 37-year-old pregnant woman with BRAF-mutated disseminated MM who was treated with the BRAF-inhibitor vemurafenib. This is the first and only described case of a patient who was treated with vemurafenib during pregnancy.

The patient was diagnosed with metastatic MM in the 22nd week of pregnancy. The treatment was initiated at week 25. Immediate response to the treatment was observed, both in terms of clinical improvement and normalization of biochemical tests. The patient experienced a 3-month PFS. Treatment with vemurafenib prolonged the duration of gestation as long as to week 30, decreasing the risk of immaturity related complications. Due to growth inhibition, more likely caused by the metastatic disease rather than the treatment, a caesarean section was performed. A healthy newborn was delivered 5 weeks after the initiation of treatment.

Discussion

Malignant melanoma occurs frequently in younger people where the BRAF mutation is more common. This skin cancer shows increasing incidence trends and vemurafenib is an established treatment for BRAF mutated advanced melanoma [71].

One of the ultimate challenges of modern oncology is anticancer treatment of pregnant patients where a crucial factor is the development of the fetus. Prematurity, and in particular severe prematurity, means risks for complications while treatment with traditional chemotherapeutics has not revealed any negative effects for the child [191]. Therefore, it is recommended that cytotoxic treatment due to a metastatic disease during pregnancy should continue, preferably until at least the 35th week of gestation in order to improve the prognosis for the premature infant.

Animal experiments indicate that vemurafenib only to a limited extent crosses the placenta and exposure of the drug was not associated with teratogenesis. In our case however, the newborn infant's serum drug concentration was markedly higher than anticipated from the preclinical studies, approximately 50% of the maternal concentration.

The child has been carefully followed by different specialists with the most recent check-up in December 2015. The only remark is that she is -2SD regarding length, weight and head measurement and this is fully explained by the early delivery.

Paper V

Case presentation

In paper V we presented the first -to our knowledge- described case of successful vemurafenib treatment of a patient with disseminated ocular melanoma. We reported a case of a 53 year-old Swedish female with a BRAF^{V600E} mutated metastatic CM. The patient had experienced disease progression on established treatments. Thus, treatment with vemurafenib was considered an option. The patient had developed brain metastases before the initiation of vemurafenib treatment. A clear correlation between the on-set of vemurafenib therapy and the regression of metastases was clinically observed. The patient benefitted from a PFS of four months.

Discussion

Conjunctival melanoma is a rare malignancy lacking an effective treatment in the advanced stage [192]. The patient clearly benefitted from the vemurafenib treatment and PFS reached the median for patients with brain-metastatic cutaneous MM. BRAF therapy is not established for patients with CM or other ocular melanomas [73]. The current report supports that treatment with a BRAF-inhibitor should be considered for CM patients with activated BRAF mutations despite the lack of clinical trials.

Concluding Remarks

The main findings of this thesis were:

- The treatment with AdCD40L was feasible and with no serious side effects.
- Decreasing intratumoural levels of IL8 post-treatment correlated to OS.
- AdCD40L therapy induced T-cell activation by increasing the Teff/Treg ratio and the levels of death receptors.
- Upregulation of serum proteins connected to immunity. Upregulation of molecules connected to interaction with DCs and T-cell trafficking and migration.
- “No responding” patients –*i.e.* with survival shorter than 6 months- had higher levels of immunosuppressive molecules.
- DW-MRI is superior to FDG-PET/CT for early prediction of tumour response, in terms of OS.
- The first described case of a pregnant woman with metastatic BRAF-mutated MM treated with a BRA- inhibitor.
- The first described case of a patient with metastatic BRAF-mutated CM experiencing benefit from vemurafenib treatment.

Future Perspectives

Immunotherapy is decades old, but it is just in recent years, a series of new immunotherapeutic agents have taken cancer care in a new era. It is a fact that recent advances in both tumour biology and immunology have led to the development of new agents that prolong PFS and OS of MM patients. Despite demonstrated successes, only a minority of patients responds to immunotherapy interventions. Translation of the knowledge gained from the biology of TME might constitute a bridge to impact on prognosis and response to therapy in MM patients in the years to come. Attempts are being made to improve immunotherapy by developing biomarkers in order to select patients for treatment and response monitoring.

CD137, a member of the TNFR superfamily, regulates the activation of CD4+, CD8+ -T, DCs, and NK-cells. The ligation of CD137 provides a costimulatory signal in a number of different immune cell subsets.

In addition, it is known that intratumoural therapies are applicable in the treatment of metastatic MM due to their local activity and the ability to trigger a systemic immune response after injection. One promising type of treatment is the use of oncolytic viruses and they can be administered intratumourally. A variety of oncolytic viruses is being tested in clinical trials and managed to demonstrate efficacy in cancer patients.

All the patients in our study were heavily pre-treated, resulting in a poor immunological capacity. Although the study comprised a limited number of patients, the risk of biological heterogeneity was high due to different types of MM. Despite of this, the results were encouraging and further studies with larger patient populations with similar types of MM are warranted. Moreover, possible improvements of the treatment schedule are desirable, for example with continuous injections including maintenance therapy and/or concomitant with other immunomodulating agents. It would be interesting to explore the efficacy of the combination of an oncolytic virus carrying the CD40L gene and an agonistic anti-CD137 monoclonal antibody.

In our third paper we have not evaluated the therapeutic effect on all metastases, because of the restricted field of view of MRI. In order to overcome this obstacle we could use a more detailed whole-body approach for DW-MRI, with more b-factors in the protocol.

I hope to continue to work with academic studies of immunotherapy and MM.

Sammanfattning på svenska

Malignt melanom (MM) är den mest aggressiva formen av hudcancer och en av de vanligaste orsakerna till cancerrelaterade dödsfall bland unga vuxna. Att utnyttja immuncellers förmåga att bekämpa tumörceller är en typ av cancerbehandling som har utvecklats de senaste åren. Studier har visat att immunsystemet hos melanompatienter kan manipuleras och aktiveras till bättre tumörkontroll. På senare tid har revolutionerande nya läkemedel i form av BRAF-, MEK-hämmare och PD1-/PDL1-hämmare har medfört att prognosen förbättrats avsevärt och att vissa patienter möjligen kan bli botade. Prognosen är dock fortfarande dålig när tumören har spridit sig. Dessutom är dessa nya behandlingar förknippade med besvärliga biverkningar, varför det är angeläget att utveckla förbättrade behandlingsstrategier.

Malignt melanom är en lovande cancerform för immunstimulerande genterapi. CD40L är en av de mest potenta stimulatorerna av immunförsvaret, finns normalt uttryckt på aktiverade immunceller och stimulerar cytotoxiska T-celler som kan angripa cancerceller. AdCD40L är ett oskadliggjort adenovirus som transporterar den immunstimulerande genen för CD40L. När AdCD40L kommer i kontakt med tumörceller tar det sig in i cellerna och levererar genen, varpå viruset självdör. Genen transkriberas i cellen till proteinet CD40L, som i sin tur sätter sig på cellytan och aktiverar omgivande immunceller. Detta aktiverar fler T-lymfocyter som angriper tumörceller.

Traditionellt har behandlingseffekten bedömts utifrån radiologiska utvärderingar och i synnerhet utifrån datortomografi (DT)-undersökningar. Immunoterapi kan dock medföra en så kallad pseudoprogres (falsk progress). Funktionella metoder har därför börjat användas för att bedöma tumörrespons. FDG-PET/DT används allt mer men metoden är dyr och ibland kan det vara svårt att skilja cancer från inflammation. Diffusionsviktad MRT (DW-MRI) avspeglar mikrostrukturen i normal och patologisk vävnad baserad på vattenmolekylernas möjlighet till spontan mikroskopisk rörelse. Olika parametrar kan beräknas och användas för att förbättra responsbedömningen.

Vi har genomfört en translationell fas I/IIa-studie med metastasinjektioner med det modifierade viruset AdCD40L. I studien ingick 24 patienter med spridd MM. Studien var indelad i tre delar. De första sex patienterna erhöll intratumourala injektioner med AdCD40L en gång i veckan, totalt fyra behandlingar (kohort 1). De följande nio patienterna erhöll en låg dos av cellgiftet cyklofosamid som konditionering innan den första och den fjärde AdCD40L-injektionen (kohort 2). I den tredje delen (kohort 3) erhöll nio

patienter dessutom strålning som en 8 Gy engångsfraktion mot den metastas som var utvald för injektionsbehandling en vecka innan den första injektionen. Cyklofosamid har i den låga dos som användes ingen cytotoxisk effekt mot cancer utan gavs för att förstärka effekten av immunterapi. Studier har visat en synergism mellan strålbehandling och immunterapi. Engångsfraktionen gavs för att förstärka immunterapi-effekten.

Resultaten visade att behandlingen var säker, med endast milda och övergående biverkningar. Vi såg att de patienter som fått både cyklofosamid och immunstimulerande genterapi med AdCD40L hade en bättre överlevnad efter sex månader än de som bara behandlats med AdCD40L. Patienterna som uppvisade bäst överlevnad hade fler aktiverade immunceller i blodet och lägre nivåer i tumörerna av en substans (IL8) som stimulerar tumörspridning. Våra analyser visar att AdCD40L framkallar T-effektorcellreaktioner och T-cellsaktivering. Inflammatoriska cytokiner och andra plasmaproteiner ändrades åt det positiva hållet av AdCD40L-behandlingen. Vidare visar studieresultaten att de funktionella DWI-parametrarna kan vara bättre tidiga prediktorer för totalöverlevnad än de som används i klinisk vardag, nämligen metaboliska och morfologiska kriterier för FDG-PET/DT respektive DT/MRI.

Resultaten är uppmuntrande och motiverar ytterligare studier. Det vore intressant att testa om fler intratumoral injektioner kunde förbättra effekten. CD40L-konceptet kan utvecklas vidare genom att använda ett onkolytiskt virus istället, som förutom CD40L även skulle uttrycka en immunstimulerande molekyl till.

Omkring hälften av alla patienter med MM har en mutation i en gen som kodar för proteinet BRAF som styr cancercellen. Mutationen leder till att cellsignaleringen blir onormal, vilket i sin tur gör att mängden cancerceller ökar och att de överlever i större omfattning. Det innebär att patienter med den BRAF-muterade formen av MM har en mer aggressiv sjukdom vilket påverkar prognosen negativt. Patienter vars melanom är BRAF positivt och som inte kan opereras eller där sjukdomen spridit sig kan behandlas med BRAF-hämmare.

Vi studerade ovanliga fall av spridd BRAF-muterat MM hos enstaka patienter eftersom den extremt låga prevalensen inte tillåter några kliniska prövningar. I avhandlingen beskrivs två fall av patienter med spridd MM som behandlades med BRAF-hämmaren vemurafenib utanför indikation.

Vi rapporterar det första beskrivna fallet i världen av behandling med BRAF-hämmare under graviditet. Man har tidigare varit restriktiv med olika cancerbehandlingar i denna situation då riskerna för barnet har ansetts som stora. Nya studier visar dock att cancerbehandling under den andra eller tredje trimestern inte nödvändigtvis är skadlig för fostret. I svåra fall bör onkologisk behandling om möjligt initieras och upprätthållas tills fostret inte är gravt underburet. Detta eftersom mycket för tidig födsel innebär en kraftigt ökad risk för bestående men. I vårt fall, en 37-årig gravid kvinna som

diagnostiserades med ett spritt BRAF-muterat MM, initierades behandlingen under andra trimestern. Vemurafenib-behandlingen var väl tolerabel och möjliggjorde en längre graviditet. Kejsarsnitt utfördes 5 veckor efter start av behandlingen och en frisk flicka föddes. Patienten avled tyvärr två månader senare.

För första gången någonsin beskrivs också ett fall med en patient med spritt konjunktivalt melanom (KM), en typ av ögonmelanom, som gynnades av behandling med vemurafenib. Patienter med spritt KM har mycket dålig prognos och immunterapi har i princip ingen effekt, så det är angeläget att hitta andra behandlingsalternativ för denna mycket ovanliga och allvarliga sjukdom. Ytterligare studier behövs för att utvärdera effekten av BRAF-hämmare i de olika subgrupperna av ögonmelanom.

Περίληψη στα Ελληνικά

Το κακόηθες μελάνωμα είναι η πιο επιθετική μορφή καρκίνου του δέρματος και μία από τις πιο κοινές αιτίες καρκίνου σχετιζόμενη με θνησιμότητα σε νεαρούς ενήλικες. Τα τελευταία χρόνια νέες θεραπείες κατά του μεταστατικού μελανώματος όπως οι αναστολείς BRAF και MEK καθώς και αναστολείς PD1/PDL1, έχουν οδηγήσει σε σημαντική βελτίωση της πρόγνωσης και σε ορισμένες περιπτώσεις ακόμη και σε «ίαση». Ωστόσο, η πρόγνωση του προχωρημένου μελανώματος εξακολουθεί να είναι σχετικά κακή. Επιπλέον, αυτές οι νέες θεραπείες συνοδεύονται από σοβαρές παρενέργειες. Είναι οπότε σημαντικό να αναπτυχθούν πιο αποτελεσματικές θεραπείες.

Η αξιοποίηση της ικανότητας του ανοσοποιητικού συστήματος να καταπολεμά τα καρκινικά κύτταρα είναι μια στρατηγική για την αντιμετώπιση του καρκίνου που έχει μελετηθεί έντονα, ιδιαίτερα τα τελευταία χρόνια. Διάφορες μελέτες έχουν δείξει ότι το ανοσοποιητικό σύστημα των ασθενών με μελάνωμα μπορεί να ενεργοποιηθεί οδηγώντας σε καλύτερο έλεγχο του καρκίνου από τον ίδιο τον οργανισμό.

Το κακόηθες μελάνωμα είναι μια μορφή καρκίνου που εξαιτίας της ανοσογονικότητας του ενδείκνυται για ανοσοδιεγερτική γονιδιακή θεραπεία, όπως αυτή που παρουσιάζεται στην παρούσα διδακτορική διατριβή. Ο CD40 Ligand (ligand=διεγέρτης, CD40L) είναι ένας από τους πιο ισχυρούς διεγέρτες του ανοσοποιητικού συστήματος. Υπάρχει στην κυτταρική μεμβράνη των ενεργοποιημένων ανοσοκυττάρων και διεγείρει τα κυτταροτοξικά T κύτταρα που επιτίθενται στα καρκινικά κύτταρα. Ο AdCD40L είναι ένας γενετικά τροποποιημένος αδενοϊός, που φέρει το ανθρώπινο γονίδιο για τον CD40L και μπορεί να διεγείρει το ανοσοποιητικό σύστημα για την καταπολέμηση του καρκίνου.

Η αξιολόγηση της ανταπόκρισης στην αντικαρκινική θεραπεία γίνεται συνήθως με απεικονιστικές εξετάσεις όπως η αξονική τομογραφία. Ωστόσο, παρατηρήθηκε ότι οι αξονικές εξετάσεις ασθενών μετά την χορήγηση ανοσοθεραπείας μπορεί να δείξουν ψευδή πρόοδο της νόσου. Για τον λόγο αυτό καταβάλλονται προσπάθειες για την δημιουργία νέων πιο αξιόπιστων απεικονιστικών τεχνικών με σκοπό τον καλύτερο προσδιορισμό της ανταπόκρισης στην ανοσοθεραπεία. Το FDG-PET/CT χρησιμοποιείται όλο και περισσότερο, αλλά είναι δαπανηρή μέθοδος και μερικές φορές μπορεί δύσκολα να διακρίνει την καρκινική βλάβη από μια απλή φλεγμονή. Η μαγνητική τομογραφία διάχυσης (DW-MRI) είναι η απλούστερη μορφή της

απεικόνισης διάχυσης και χρησιμοποιείται ολοένα και περισσότερο για την μελέτη των όγκων. Η DW-MRI είναι μια ακολουθία παλμού ευαίσθητη ως προς την τυχαία κίνηση των μορίων του ύδατος μέσα στους ιστούς (Brownian motion). Η διάχυση είναι μια τρισδιάστατη διαδικασία σε μικροσκοπικό επίπεδο, που ποικίλλει στις διάφορες διευθύνσεις. Η διάχυση των μορίων του ύδατος των ιστών δεν είναι τυχαία και ελεύθερη αλλά περιορίζεται από την παρουσία μεμβρανών, νευρικών ινών και εξωκυττάρων μακρομορίων. Μπορούν να υπολογιστούν και να χρησιμοποιηθούν διαφορετικές παράμετροι για την αξιολόγηση της απάντησης του ασθενούς στην θεραπεία.

Στα πλαίσια της κλινικής μας μελέτης δόθηκαν ενέσεις με τον τροποποιημένο ιό AdCD40L κατευθείαν σε μεταστάσεις. Η μελέτη περιελάμβανε 24 ασθενείς με μεταστατικό κακόηθες μελάνωμα. Οι πρώτοι έξι ασθενείς έλαβαν ενδομεταστατικές ενέσεις με AdCD40L μία φορά την εβδομάδα (συνολικά τέσσερις ενέσεις). Οι επόμενοι εννέα ασθενείς έλαβαν χαμηλή δόση του χημειοθεραπευτικού κυκλοφωσφαμίδη πριν από την πρώτη και την τέταρτη ένεση με AdCD40L. Οι τελευταίοι εννέα ασθενείς έλαβαν πρώτα ακτινοβολία (8Gy) στην μετάσταση που είχε επιλεγεί για την ένεση. Είναι γνωστό ότι η κυκλοφωσφαμίδη παρόλο που δεν έχει καμία αντικαρκινική δράση όταν χορηγείται σε χαμηλή δόση ενισχύει την δράση της ανοσοθεραπείας. Μελέτες έχουν δείξει πιθανή συνέργεια μεταξύ της ακτινοβολίας και της ανοσοθεραπείας. Η ακτινοβολία που χορηγήθηκε στα πλαίσια της έρευνάς μας δόθηκε με σκοπό την ενίσχυση της δράσης της ανοσοθεραπείας με τον AdCD40L.

Τα αποτελέσματα μας έδειξαν ότι η θεραπεία ήταν ασφαλής, με ήπιες και παροδικές παρενέργειες. Οι ασθενείς που έλαβαν χαμηλή δόση κυκλοφωσφαμίδης και ενέσεις με AdCD40L είχαν καλύτερη 6-μηνια επιβίωση σε σύγκριση με τους ασθενείς που έλαβαν μόνο ενέσεις. Οι ασθενείς με την πιο μακρά επιβίωση είχαν σημαντικά μεγαλύτερο αριθμό ενεργοποιημένων ανοσοκυττάρων και ταυτόχρονα χαμηλότερα επίπεδα της ουσίας IL8 που ευνοεί την ανάπτυξη του καρκίνου. Οι αναλύσεις μας επιβεβαίωσαν ότι η θεραπεία με AdCD40L προκαλεί ενεργοποίηση αυτού του συγκεκριμένου τύπου T κυττάρων που επιτίθενται στα καρκινικά κύτταρα. Διάφορες κυτοκίνες καθώς και άλλες πρωτεΐνες πλάσματος τροποποιήθηκαν θετικά μετά την αγωγή με Ad4040L. Τα αποτελέσματα έδειξαν ότι οι DWI παράμετροι μπορεί να είναι καλύτεροι πρόωμοι προβλεπτικοί παράγοντες για την συνολική επιβίωση σε σύγκριση με το PET/CT και την αξονική τομογραφία καθώς και την απλή μαγνητική τομογραφία.

Τα ανωτέρω ελπιδοφόρα αποτελέσματα δικαιολογούν τον σχεδιασμό νέων κλινικών ερευνών που αφορούν την ενεργοποίηση του CD40L. Για παράδειγμα θα μπορούσε να εξεταστεί εάν συνεχιζόμενες ενέσεις στις μεταστάσεις θα οδηγούσαν σε βελτίωση της κατάστασης του ασθενούς. Η

αν η αντικατάσταση του αδενοϊού με έναν ογκολυτικό ιό που θα έφερε εκτός του CD40L και ένα ακόμα ανοσοδιεγερτικό μόριο πχ το CD137.

Περίπου το 50% των ασθενών με μελάνωμα παρουσιάζουν μία σωματική μετάλλαξη, την BRAF^{V600E}. Υπάρχει ένας BRAF αναστολέας, η vemurafenib, που επιτυγχάνει σημαντική ανταπόκριση του όγκου καθυστερώντας την εξέλιξη της νόσου και αυξάνοντας τη συνολική επιβίωση στους θετικούς για τη μετάλλαξη BRAF^{V600E} ασθενείς.

Στα πλαίσια αυτής της διδακτορικής μελέτης έλαβε χώρα η παρακολούθηση δύο σπάνιων περιστατικών προχωρημένου μελανώματος. Είναι σημαντική η καταγραφή τέτοιων περιστατικών εξαιτίας του γεγονότος ότι είναι πρακτικά αδύνατη η διεξαγωγή μεγάλων κλινικών δοκιμών λόγω της σπανιότητάς τους.

Η πρώτη περίπτωση, που είναι και η πρώτη αναφορά παγκοσμίως, αφορά μια έγκυο γυναίκα ηλικίας 37 ετών που διαγνώστηκε με μεταστατικό κακόηθες μελάνωμα θετικού για τη μετάλλαξη BRAF^{V600E}, κατά την διάρκεια του 2^{ου} τριμήνου της κύησης. Είναι σχετικά ασφαλής η χορήγηση χημειοθεραπείας κατά την διάρκεια του 2^{ου} και του 3^{ου} τριμήνου κύησης σύμφωνα με τις μελέτες των τελευταίων ετών. Ποτέ πριν δεν είχε χορηγηθεί vemurafenib σε έγκυο ασθενή. Η απόφαση για χορήγηση του BRAF αναστολέα ελήφθη σε μια προσπάθεια επιμήκυνσης της διάρκειας της κύησης ώστε να αποφευχθεί πρόωρος τοκετός. Η ασθενής απάντησε θετικά στην θεραπεία, που είχε σαν αποτέλεσμα τη γέννηση με καισαρική ενός υγιέστατου κοριτσιού. Η ασθενής απεβίωσε λίγες βδομάδες μετά την γέννηση εξαιτίας της ανεξέλεγκτης προόδου της νόσου.

Για πρώτη φορά περιγράφεται μια περίπτωση ασθενούς με μεταστατικό μελάνωμα του επιπεφυκότα θετικού για τη μετάλλαξη BRAF^{V600E} που επωφελήθηκε από την χορήγηση της vemurafenib. Το μελάνωμα του επιπεφυκότα είναι ένας σπάνιος τύπος οφθαλμικού μελανώματος με εξαιρετικά κακή πρόγνωση όταν είναι προχωρημένου σταδίου. Οι νέες θεραπείες κατά του μελανώματος του δέρματος είναι ελάχιστα έως καθόλου αποτελεσματικές στην θεραπεία του οφθαλμικού μελανώματος. Η 53χρονη ασθενής είχε ήδη λάβει όλες τις αντικαρκινικές θεραπείες που υπήρχαν διαθέσιμες χωρίς αποτέλεσμα. Η ασθενής παραπέμφθηκε στην Ογκολογική Κλινική του Πανεπιστημιακού Νοσοκομείου της Ουψάλας όπου και πραγματοποιήθηκαν διάφορες εξετάσεις. Μια από αυτές ήταν και για την ύπαρξη μετάλλαξης BRAF η οποία δεν είχε πραγματοποιηθεί καθώς δεν αποτελεί εξέταση ρουτίνας για το οφθαλμικό μελάνωμα. Η απάντηση στην θεραπεία με vemurafenib ήταν ραγδαία παρά την ύπαρξη ακόμη και εγκεφαλικών μεταστάσεων κατά την έναρξη της θεραπείας. Σχεδόν 4 μήνες μετά, η θεραπεία έπαυε να είναι αποτελεσματική. Είναι επιτακτική η ανάγκη για την ανακάλυψη νέων θεραπειών για τους διάφορους τύπους του οφθαλμικού μελανώματος.

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