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IMMUNOLOGICAL ALTERATIONS, THERAPIES WITH IMMUNE CHECK-POINT INHIBITORS AND BEYOND IN PATIENTS WITH METASTATIC MELANOMA

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Immunological alterations, therapies with immune check-point inhibitors and beyond in patients with metastatic melanoma
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“SOME PEOPLE FEEL THE RAIN, OTHERS JUST GET WET”

Bob Marley

To what I come from,

to all people who matter deeply to me,

to my wonderful daughter and her life

ABSTRACT

In the last decade the treatment of metastatic melanoma (MM) has been revolutionized and changed the life of many patients, offering prolonged survival and improved symptom control. These treatment strategies have also changed the way we treat malignancies in general and the treatment of melanoma has been the role model for new treatment strategies.

Even though there has been a revolution that we have been part of, this revolution comes with responsibilities as many patients do not benefit from the new treatments and many patients experience toxicity. One key responsibility is to broaden our knowledge about tumor immunology and immunotherapy and to identify predictive biomarkers that allow for preselection of patients in order to identify patients who benefit from specific therapies and spare other patients from treatments, with side effects, that they will not benefit from. In addition, we have a societal and an ethical responsibility, as the treatments are costly and some of the side effects result in prolonged hospitalizations and lowered quality of life for the patients affected. We also have a responsibility not to be totally blinded by the success of the newly approved therapies, but to be open-minded and keep other well documented therapies in mind when recommending treatment strategies.

As many new treatments target the immune system, the chance to identify predictive biomarkers most likely lies within studies of the immune system before, during and after treatment, to see if changes in different immune cell compartments can be related to response and toxicity.

The overall aim of this thesis is to find biomarkers that correlate with response and toxicity to therapy with immune check-point inhibitors (ICI) and to study mechanisms in the immune system, in addition to those related to T-cells, during treatment with anti-CTLA 4 and anti-PD1 antibodies. In addition, we conducted a phase I trial with adoptive T cell therapy (ACT) with or without dendritic cell (DC) vaccination, with the intention to explore an additional safe and effective treatment strategy to patients progressing on or not responding to immune checkpoint inhibitors (ICI).

In paper I and II we performed comprehensive immune monitoring of patients treated with ICI; in paper I with anti-CTLA-4 antibodies and in paper II with anti-PD-1 antibodies. In paper I we observed that patients benefitting from treatment with anti-CTLA-4, and experiencing prolonged overall survival, had decreasing monocytic myeloid-derived suppressor cells (MDSCs) during treatment. It was also observed that CD8+ effector memory T cell frequencies at the end of treatment were higher in patients with clinical benefit and correlated with longer survival. In addition to this, it was observed that in patients experiencing toxicity from treatment there was a correlation between the amount of eosinophil granulocytes and the onset of toxicity. In paper II it was observed that two distinct cell types could be correlated with overall survival, neutrophil granulocytes and MDSCs. In

addition, it was observed that patients experiencing long progression free survival (PFS) had low frequency of CD69+ natural killer (NK) cells and low frequency of monocytic MDSCs at baseline.
In paper III we could state that it was feasible and safe, with limited toxicity, to conduct a clinical trial with ACT with or without DC vaccination. In addition, we observed that four out of five patients treated with ACT and DC vaccination responded to the treatment.

It is of great importance to find predictive biomarkers that could offer pre-selection of patients and the data shown in paper I-II suggest that some of the explored markers could be implemented in a clinical setting, but need to be further validated in prospective clinical studies. In paper III it was shown that ACT with DC vaccination, as a novel treatment is safe, brings benefit to patients and could be offered to a limited number of appropriately selected patients.

LIST OF SCIENTIFIC PAPERS

I. **Ipilimumab treatment decreases monocytic MDSCs and increases CD8 effector memory T cells in longterm survivors with advanced melanoma**
Yago Pico de Coana**, Maria Wolodarski**, Isabel Poshke, Yuya Yoshimoto, Yuan Yang, Maria Nyström, Ulrika Edbäck, Suzanne Egyhazy Brage, Andreas Lundqvist, Giuseppe Masucci, Johan Hansson, Rolf Kiessling
Oncotarget, 2017, Vol 8, pp: 21539-21553

II. **PD-1 checkpoint blockade in advanced melanoma patients: NK cells, monocytic subsets and host PD-L1 expression as predictive biomarker candidates**
Yago Pico de Coana, **Maria Wolodarski**, Irene van der Haar Ávila, Takahiro Nakajima, Stamatina Rentouli1, Andreas Lundqvist, Giuseppe Masucci, Johan Hansson, Rolf Kiessling
OncoImmunology,2020, vol 9, NO.1, 1-14

III. **Complete and long-lasting clinical responses in immune checkpoint inhibitor-resistant, metastasized melanoma treated with adoptive T cell transfer combined with DC vaccination**
Tanja Lövgren*, **Maria Wolodarski***, Stina Wickström*, U. Edbäck , E. Martell, K.Markland, P. Blomberg, M. Nyström, A. Lundqvist, H. Jacobsson , G. Ullenhag, P. Ljungman, J. Hansson, G. Masucci, R. Tell , I. Poschke, L. Adamson, J. Mattsson, R. Kiessling
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CONTENTS

LIST OF ABBREVIATIONS

ACT	Adoptive cell therapy
ADCC	Antibody-dependent cellular cytotoxicity
AE	Adverse event
AJCC	American Joint Committee on Cancer
APC	Antigen presenting cell
Arg1	Arginase-1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CR	Complete response
CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T-lymphocyte antigen -4
DC	Dendritic cell
FDA	US Food and Drug Administration
Gp100	Glycoprotein 100 or melanocyte protein
ICOS	Inducible T-cell costimulator
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN-γ	Interferon gamma
IL-2	Interleukin-2
ICI	Immune check-point inhibitors
LDH	Serum lactate dehydrogenase
MHC	Major histocompatibility complex
MAGE-A3	Melanoma-associated antigen 3
MAPK	Mitogen-activated protein kinase pathway
MART-1/MelanA	Melanoma antigen recognized by T cells
MDSC	Myeloid Derived Supressor Cells
MMR	Mismatch repair
MR	Mixed response
MSI	Microsatellite instability
NK cells	Natural Killer cells
NLR	Neutrophil to Lymphocyte Ratio
NY-ESO-1	Cancer/testis antigen 1

iNOS	inducible NO synthase
ORR	Overall response rate
OS	Overall survival
PD-1	Programmed death-1
PFS	Progression free survival
PR	Partial response
PTEN	Phosphatase and tensin homolog
RFS	Relapse free survival
RLC	Relative lymphocyte count
ROS	Reactive Oxygen Species
SCC	Squamous cell carcinoma
TAA	Tumor-associated antigen
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TIL	Tumor infiltrating lymphocyte
TMB	Tumor mutational burden
TME	Tumor micro environment
TNF-α	Tumor necrosis factor –alfa
TNM	Tumor Node Metastses
TSA	Tumor-specific antigen
UV	Ultra violet
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 BACKGROUND

In the last decade the treatment of melanoma has changed enormously. When I was a junior oncologist around the year of 2006, working in the clinical ward, I remember the often rather young patients with metastatic melanoma having the stormy course of disease that only melanoma can have. From being almost free of symptoms from the disease they deteriorated very rapidly and succumbed in a very brief time, often leaving families with many questions and unsolved matters.

The disease itself is rather mysterious, originating from a tiny spot where sunlight, which people here in the north seek so much, plays a major role with UV radiation being the most important external risk factor [1]. This light that many of us yearn causes cellular and biological effects which result in DNA damage and mutations, cellular growth stimulation and effects on the immune system and inflammation, that can cause cancer, which, if it is not dealt with in time, can be a potentially deadly disease.

1.1.1 Terminology

The word melanoma originates from the greek “melas” meaning dark and “oma” meaning process/tumor (see below).The word melanoma was earlier used in a broader sense to describe any melanocytic tumor, but today the narrower sense only referring to malignant types has become dominant.

1.1.2 A brief early history of melanoma

In approximately the 5th century BC, Hippocrates was the first to record a description of melanoma [2]. As early as in the middle of the 17th century a British surgeon, Highmore, among others referred to a condition of “fatal black tumors with metastases and black fluid in the body” [2]. After that, descriptions of melanoma are rare until the middle of the 18th century when the surgeon John Hunter was recorded to be the first to perform surgery for melanoma on a patient [2]. In the early 19th century a French physician, Rene Laennec was the first to describe melanoma as a disease. He had observed dark lesions in multiple organs of patients and he described the condition, with similarity to Hippocrates’s description, as melanosis, although it was the pathologist Carswell who re-introduced the term melanoma since Hippocrates [2]. After that an English general practitioner, William Norris, was the first to study melanoma more in detail and to make correlations between pathology, epidemiology and management of melanoma [3]. In the 19h century the knowledge about melanoma progressed and guidelines for surgical treatment of melanoma took form [2].

1.1.3 The puzzling behavior of the disease

Melanoma is also special in the way that it is both frequently affected by the immune system and in turn can affect the immune system in a negative way. Spontaneous regression, both of the primary lesion and, more rarely, even of metastatic disease can be seen and this is reported to be more frequent for melanoma than for other malignancies [4]. The cancer can hide from the immune system after primary surgery, lay dormant for many years, then suddenly reappear and imitate other types of ailments or diseases, deceiving the patient and clinicians. Not seldom do clinicians meet patients who had their primary lesion removed many years ago, making them forget they even had a malignancy. Then something happens. May it be one of those rather soft lumps under the skin that are so easily taken for lipomas or may it be a few of those reddish dots that could be taken for rash or eczema or may it be those black-blue dots marking the skin in their distinct way. Or just that feeling that something has changed, that you feel more tired, or that you have a kind of pain that is difficult to put a finger on. These patients have often wandered through many contacts with healthcare, due to their diffuse symptomatology, and often after a rather long period it becomes clear that it is their previous melanoma that has come back and spread.

A relative of mine experienced melanoma in this way in the beginning of this century and sometimes the question is asked: what if? What if he had gotten the disease back today? Would he have been cured then? Could he have lived now but with a chronic disease? If the new treatments had been used in the clinic earlier, would he have responded? No one can tell but it makes one think how much has improved in the treatment of advanced disease and in what short period of time it has happened. In my lifetime up to now, the regimens of handling this disease have changed enormously and only during my time as practicing oncologist have there been tremendous changes.

1.1.4 Epidemiology

My relative got his primary lesion in the late 1970s or early 1980s. Since then the incidence of melanoma has increased very much: in the past decade, based on the age standardized incidence, there has been an increase with around 5 % per year for both women and men in Sweden [5]. Sweden is one of the countries in Europe with the highest incidence of melanoma [6]. In the Nordic countries the incidence is the highest in Denmark, Norway and Sweden but remarkably lower in Finland and Iceland [7].
The increase in incidence may be related to changes in life-style regarding sun exposure, with many more people seeking sun exposure also during winter time with holidays abroad and through indoor tanning in beauty salons [8].Today, about 4000 people per year in Sweden are diagnosed with primary invasive melanoma and it is the sixth most common malignancy among men and the fifth among women [9].The relative of mine lived in Karlstad, Värmland just below the middle of Sweden and the incidence also varies with latitude and is almost the double in the south of Sweden compared to the north of Sweden [10]. He was in his late 30s/beginning of his 40s when diagnosed and today the median age of diagnosis is 69 years among men and 65 years among women [11]. The lesion he had was

localized over one of the breasts and there is a difference between the genders regarding most common localization; for men it is more common on the trunk and for women more common on lower extremities [11].

1.1.5 The primary lesion

Like for many others a close kin, his wife, observed a lesion that she thought had changed and that she thought stood out, an “ugly duckling”. In three of four cases of melanoma it is the patient him-/herself or a relative that notes the lesion [12]. It has been shown that male individuals living alone tend to seek healthcare more rarely and tend to present with thicker and more advanced primary lesions [13]. It is also shown that if the primary lesion is discovered by a physician, regardless of specialty, it is thinner than if the patient him-/herself discovers it [14]. Before my relative’s lesion was removed some time passed due to the fact that physicians did not consider that it looked atypical. However, anamnestic information about change and symptoms are important, even though the lesion itself seems to be unspecific, and should be guiding in making decision on further investigation [15]. In 20-30% of cases of melanoma, the patient has turned to healthcare at several occasions regarding the lesion before it becomes removed, due to the difficulty to clinically diagnose a melanoma because of their vast variation in clinical presentation [16]. After having it removed and analyzed it was clear that it was a malignant melanoma that my relative had had.

1.1.6 Pathology

Melanoma is caused by malignant transformation and proliferation of melanocytes in the basal layer of the epidermis.

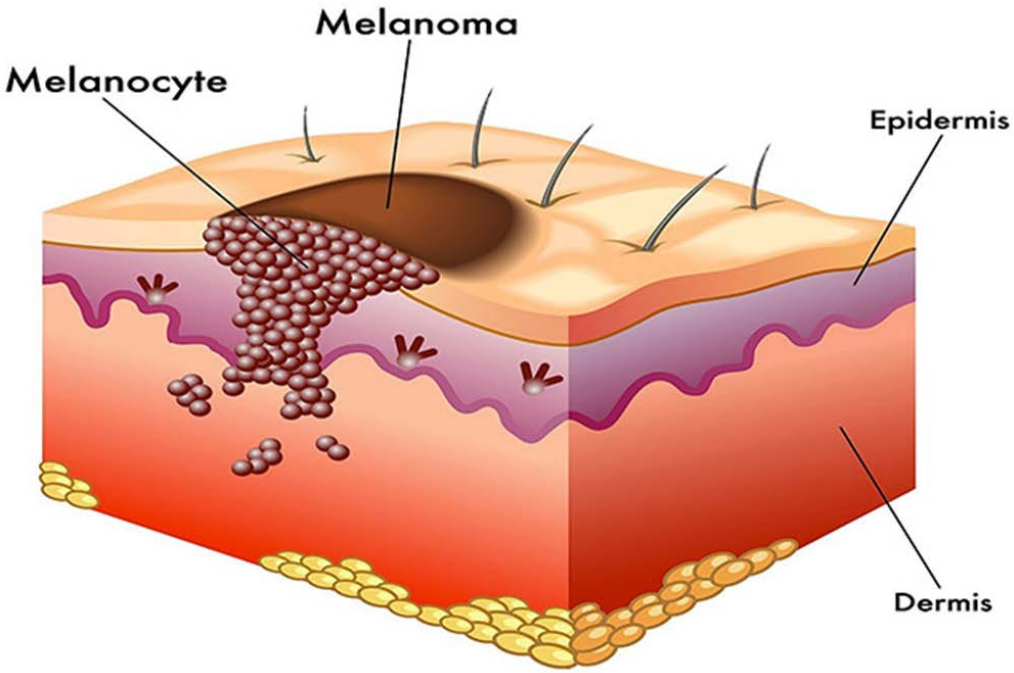

Figure 1. Melanoma is caused by malignant transformation and proliferation of melanocytes (the cells producing melanin, the pigment, which gives rise to the color of the skin) in the basal layer of the epidermis. Can Stock Photo Inc.

Earlier it was thought that the development of a malignant lesion went stepwise from a benign naevus through a dysplastic naevus to finally a malignant melanoma. In later years another theory has emerged; that melanocyte stem cells transform to melanoma stem cells, giving rise to melanoma without a precursor lesion [17]. This is supported by the fact that almost 70 % of melanomas occur de novo, without a precursor lesion such as a naevus [18][19].

Melanoma tumors contain a large number of mutations, especially melanomas that occur on skin that has been exposed to excessive UV-radiation [20]. These mutations cause activation of many signaling pathways in the cells [21]. Several types of gene mutations are common, for instance activating mutations in genes such as *BRAF* or *NRAS* or loss of or inactivation of tumor suppressor genes such as *CDKN2A* (cyclin-dependent kinase inhibitor 2A) or *PTEN* (Phosphatase and tensin homolog). The most common mutation is the *BRAF*V600 mutation which also occurs early during melanoma development. *BRAF* mutation is present in about 40-50% of cutaneous melanomas and it drives tumor progression through constitutive activation of the mitogen-activated protein kinase (MAPK) pathway [22].

1.1.7 Riskfactors

Why did my relative get a melanoma, did he have any particular risk factors?
The individual risk for melanoma is affected both by genetic and external factors such as sun habits [1][23, 24]. No one else in his family had had melanoma, but in 5-10% of the cases of cutaneous melanoma there is heredity for the disease [25]. He was fair skinned and had fair somewhat reddish hair, some freckles and rather few moles and most likely he had been burned in the sun earlier in life. In the end of the 1950s Henry Lancaster made the initial connection between ultraviolet radiation from exposure to sunlight and increased incidence on melanoma. This idea was supported by the further work of Lancaster and Nelson, who demonstrated that the characteristics of the skin had an impact on melanoma development, including skin color, texture, hair color, eye color and reaction to the sun [26].
Risk factors should be weighed together in a general risk evaluation for each individual, where rare genetic alterations can contribute to a much higher risk than more common factors such as hair color or being burnt in the sun [27].

1.1.8 Treatment of primary disease

After verification that the lesion was melanoma, my relative was operated again with local wide excision, but as far as I know no lymph node exploration. I remember his scar, a horizontal one right over one of the pectoral areas, a very distinct sign that was very obvious the times he joined us cousins for swimming.

Already in the 1890s, Herbert Snow expressed the benefits of removing the tumor and surrounding glands as a method of prophylaxis. He believed that the excision of the tumor alone was an ineffective treatment and whenever possible the draining lymph glands should

be removed [2, 28]. In the very beginning of 1900s, William Handley analyzed the lymphatic spread of secondary melanoma on a woman's leg, which formed the basis of a case study of the disease. He suggested that the surrounding subcutaneous tissue and lymph nodes should be removed [2]. Even today these observations and theories influence the primary treatment and also the follow-up guidelines, even though the width of the wide excision has varied over time as well as the opinion on how much of lymphoid glands should be removed.
Today all invasive melanomas are treated surgically with wide local excision, the width depending on the thickness of the primary tumor [29].
Sentinel node biopsy, which was introduced in the beginning of the 1990s, is today recommended for melanomas thicker than 1 mm (T2-T4 tumors, for classification see below) [30]. Sentinel node is by definition the first lymph node that drains the skin area where the primary tumor is localized and is thus the best place to selectively look for metastases [31]. Sentinel node status is, in addition to tumor thickness, a strong prognostic factor [32-34], but the biopsy has no therapeutic value in itself. Lymph node exploration is performed only if sentinel node shows periglandular/pericapsular growth, if the patient is immune suppressed, if there are microsatellites around the primary and if there are more than tree positive sentinel nodes. This is based on two large trials, DeCOG-SLT and MSLT-2, both showing that there is no benefit to perform lymph node exploration due to positive sentinel node only [34, 35].

1.1.9 Classification, staging and follow-up

Melanomas are classified according to the TNM system [36] and staged according to AJCC (American Joint Committee on Cancer) [37].

The follow-up guidelines in Sweden are based on the classification above. This follow-up system has changed during the years, only since I started working as an oncologist. Back then, almost ten years ago, the follow-up system was more intense with much more frequent clinical check-ups. But as no survival benefit has been demonstrated by this and due to the fact that around 70% of the recurrences of melanoma are discovered at other time-points than at follow-up appointments (of asymptomatic patients) [38, 39], this has changed and the clinical check-ups are much less frequent today.

1.1.10 Prevention; primary and secondary

After the surgical treatment I recall that my relative very seldom spent time in the sun, often sought the shade and that he often wore a T-shirt on the beach, which for me as a child was very mysterious. His behavior was correct. When having had one melanoma, the risk of developing a new one is increased compared to someone that has not had melanoma [40].
I remember more senior colleagues telling me about the 1990s when they travelled around Sweden staying in caravans visiting beaches to inform people on the beach of the risks of the sun, primary prevention. They told me about that period with something warm in the voice, that it had been a special time in their work-life and in their lives. Studies show that Swedes tend to sunbathe with the intention of getting tanned and that our beauty ideal is more tanned than in other European populations [41]. We Swedes also tend to protect ourselves less in the

sun and we burn more often than other nationalities [42]. Sun exposure and sun-burn early in life seems to be a special risk factor [43]. Sun exposure in childhood also affects the risk of developing many moles, which in turn is considered an increased risk factor [44]. It makes one wonder, with the increasing incidence of melanoma, if the “summer tours” should be taken into action again.

1.1.11 Prognosis

1.1.11.1 Prognosis in early stages

My relative was cured from his primary melanoma. Sadly his melanoma recurred. What was the risk of that and could it have been prevented had he only acted or behaved differently?

The survival after having a melanoma depends on several factors, both clinical and histopathological, such as, age, gender, thickness of the tumor according to Breslow and whether the tumor had an ulceration [45] [13, 46].
The prognosis in early stages of cutaneous melanoma is very good and almost all patients are cured after primary surgery with wide local excision.
The melanoma specific 5-year survival for T1 (0-1 mm thickness according to Breslow) melanomas is about 95%, but with increasing thickness the survival worsens to between 50 to 75% for T4 tumors (>4 mm thick according to Breslow). The 5 year survival for stage III melanoma with regional lymph node metastases ranges between around 40%-around 80% [47].
There is nothing that the patient self can do in order to prevent the recurrence of disease, a question that often arises when patients come for information about relapsed disease. There is no type of food known to be beneficial or no certain way of living that could prevent the disease from recurring.

1.1.11.2 Prognosis in advanced stage

Samuel Cooper was the first to formally acknowledge in the 1840’s that advanced melanoma was untreatable and that “the only chance for benefit depends on early removal of the disease” [2]. Even today this statement remains true to a great extent.
Stage IV melanomas as a group has until recently had very poor prognosis, with a one year survival around 25 %, a two year survival around 10 % and a median overall survival about six to ten months [45]. Regarding the subtypes of stage IV melanoma, according to AJCC, there are differences in survival. Patients with distal metastases to the skin, soft tissue or distal lymph nodes (M1a) have the best overall survival if the serum lactate dehydrogenase (LDH) level is normal. LDH is an enzyme that catalyzes the conversion of lactate to pyruvate and back and it is found in almost all living cells. It is expressed to a large extent in body tissues and is released if there is tissue damage. Many cancers can elevate the LDH level. Patients with metastatic disease spread to the lungs (M1b) have an intermediate prognosis. If the disease has spread to any other organs and/or if the patient has an elevated LDH (M1c) and patients with brain metastases (M1d) have the worst prognosis [36, 37]. LDH level is

also a predictor of survival and elevated LDH at diagnosis of stage IV disease means a shorter survival than if LDH is normal [48]. The 1–2-year survival is between 40–60% in patients with normal LDH, compared to between 18–32% in patients with elevated LDH levels [37]. The number of distant sites involved is also a strong predictor of survival [49], but not included in the staging system.

1.2 THE IMMUNE SYSTEM AND IMMUNE RESPONSES TO TUMOR

One can wonder what went on in my relative's body during these years before the primary melanoma occurred and was removed in the beginning of the 1980s until the disease recurred again. For certain his immune system was involved, both before the primary lesion was observed and removed, during the time he was free of disease, as well as when the disease had spread.

The immune system is the defense system which protects individuals from dangers. To do so it utilizes a diversity of cells, molecules, and organs. In its simplest form, the immune system functions in the way that it identifies and eliminates dangerous elements. It requires two necessary activities: recognition of a danger agent or antigen by certain receptors on the surface of immune cells and, once the danger element is detected, it carries out effector responses, involving a variety of cellular behaviors that protect the individual [50, 51]. Communication between cells in the immune system occurs both through cell to cell contact, as well as through chemical signals that are secreted from one cell and received by others [50, 51].

1.2.1 Division of the immune response; innate and adaptive

The immune system can in a simple way be classified into two responses; the innate immune response and the adaptive immune response. The immune response to invasion by pathogen or tumor depends on collaboration and coordination of activities in both the innate and the adaptive immune system [51].

1.2.1.1 The innate immune response

The innate immune system appeared early in animal evolution, as an essential response to infection [52]. It is often called the "more primitive" immune system which provides more immediate and somewhat more generalized defense and can be divided into immediate and induced components. The immediate innate immune system consists of epithelial barriers, mucus, enzymes and peptides that can be secreted by specific cells involved in this line of defense. The induced innate immune system is composed of white blood cells: granulocytes, macrophages, dendritic cells and Natural Killer (NK) cells, with phagocytic, cytotoxic and secretory activity. The innate immunity detects and destroys most microorganisms that enter the body within hours or even minutes. Its receptors recognize features that are common for many invading organisms and therefore it is very effective in discriminating between self and non-self. Furthermore, the innate immune response is one of the main drivers needed to activate the adaptive immune system [50, 51].

1.2.1.2 The adaptive immune response

The adaptive (or acquired) immune response evolved in early vertebrates and is much more specialized with the capacity of stronger immune response as well as immunological memory [53]. The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called antigen presentation.

The adaptive immune system is composed by B and T lymphocytes derived from hematopoetic cells in the bone marrow [54]. B cells are involved in humoral response and T cells in cell-mediated response. Both B and T cells use specialized receptor molecules that recognize antigens in a very specific way. B cells produce immunoglobulins that recognize antigens in extracellular spaces. Immunoglobulins can either be bound to the membrane of the cell or secreted. T cells recognize antigens only after they have been processed by an antigen presenting cell (APC) and presented by "self" receptor on the APC’s surface, the major histocompatibility complex (MHC) [50, 51].

The adaptive immune response is dependent on the innate for its activation and weak adaptive immune response to tumors could partly be caused by weak innate immune activation [51].

1.2.2 Immune cells and activities of importance in cancer immunity

The cells of the immune system are a collection of blood cells which can be grouped into the major categories lymphocytes (T cells, B cells, NK cells) and myeloid cells (antigen presenting cells, macrophages, dendritic cells and granulocytic cells) [50, 51].
These cells are derived from a common hematopoetic stem cell in the bone marrow.

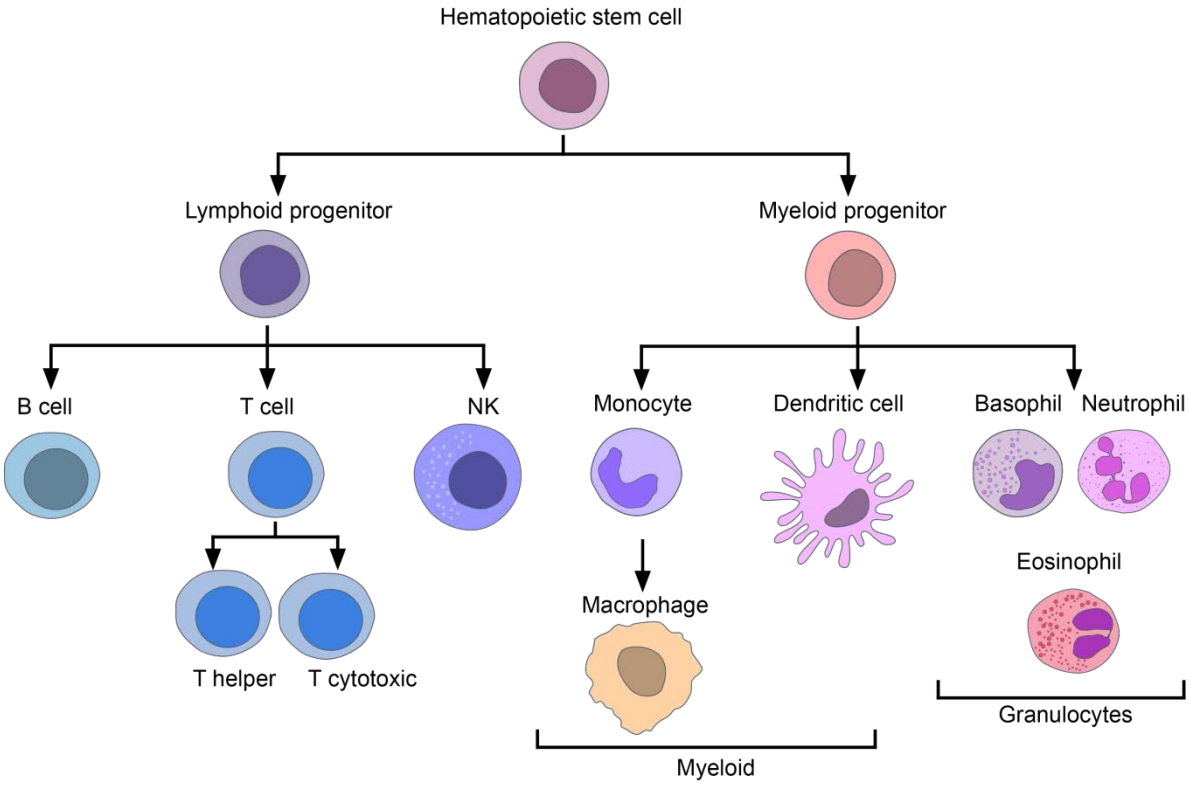

Figure 2.Major cells of the immune system

Cells involved in antitumor immunity are cells involved both in the innate and adaptive immune response and the most important ones are lymphocytes, especially B cells, T cells, NK cells and antigen presenting cells. Macrophages and dendritic cells could be said to serve as bridges between the two branches of defense.

1.2.2.1 Antigen presenting cells (APC)

Antigen-presenting cells are crucial for effective adaptive immune response, as the functioning of both cytotoxic and helper T cells are dependent on APCs. Antigen presentation is the link between innate and adaptive response. It is also involved in defense against tumors. One major task of the antigen presenting cells is to scan the body for foreign agents and internalize them, degrade them into small parts and then present them to the T cells. Almost all cells can present antigen in some way. Professional antigen-presenting cells: macrophages, B cells and dendritic cells present foreign antigens to helper T cells using MHC class II,

while all other nucleated cells use MHC class I to present antigens originating inside the cell to cytotoxic T cells.

As mentioned above, antigen-presenting cells can be categorized in two major groups; non-professional and professional.
Non-professional APCs include all nucleated cells in the body. They utilize MHC class I molecule on their surface to present peptides originated from inside the cell, in contrast to the professional APCs, which present exogenous peptides through MHC class II. CD8+, Cytotoxic T cells are able to interact with the endogenous peptide presented in the MHC class I [55].
Professional APCs express MHC class II molecules along with co-stimulatory molecules and pattern recognition receptors and they specialize in presenting antigen to T cells (CD4+) [56]. Professional APCs are very efficient at internalizing antigens, either by phagocytosis or by receptor-mediated endocytosis and processing the antigen into peptide fragments and then displaying those peptides, bound to the class II MHC molecule, on their membrane [56]. Professional APCs include dendritic cells, macrophages and B cells. In antitumor immunity, dendritic cells are considered the most potent APCs as they express high levels of co-stimulatory molecules (such as CD80 and CD86) and have a large surface area and express high levels of MHC [50, 51].

1.2.2.2 T cell activation

The most important lymphocytes in antitumor immunity are T cells and they are named for thymus, where they mature in humans. Progenitor T cells are derived in the bone marrow and they migrate to the thymus where they develop.

Thymoctes are generated in the thymus where they generate a unique T cell receptor, TCR, a complex containing the CD3 co-receptor that can be used as a T cell marker. In order to recognize and destroy only invading pathogens and their antigens, the thymocytes undergo both positive and negative selection during their development in the thymus. Positive selection is the first step, in which only thymocytes that interact with MHC I or II survive. This process ensures that the selected T cells will have an MHC affinity to be able to interact with MHC and peptide complexes to carry out immune responses which are important functions for the host [57]. Most thymocytes are eliminated by apoptosis during positive selection. Those that survive positive selection go on to the negative selection process where thymocytes that are capable of strongly binding with "self" MHC peptides undergo apoptosis. This protects the host from having T cells that are capable of reacting to “self” molecules and thereby contribute to autoimmune reactions. This process is an important component of central tolerance. About 98% of thymocytes die off during the processes of positive and negative selection in the thymus. The remaining 2% that survive the selection process leave the thymus as immunocompetent naïve T cells. Already in the thymus CD4+ and CD8+ T cells are selected, but undergo further differentiation in the periphery to specialized cells which have different functions (see below).

Naïve T cells, after development in the thymus, recirculate to lymph nodes and then back to the bloodstream every 12 to 24 hours. This high speed of circulation results in a better chance that a T cell will meet the appropriate antigen. Naïve T cells enter lymph nodes through specialized regions and thereafter travel through regions of dendritic cell networks in order to meet the appropriate antigen. If a naïve T cell does not meet and bind to any of the MHC/antigen complexes it meets, it leaves the lymph node back to the bloodstream. If the naïve T cell instead meets the appropriate antigen presenting cell that expresses the right MHC/antigen, an activation program will be started [51].

In order to be able to perform their effector functions, T cells have to be activated. The activation is a complex process which was initially described in 1970 as a two-step process [58]. Antigen presenting cells (APC) process antigens from damaged tissue and they migrate to lymph nodes where they present these antigens to T lymphocytes. The T cell receptor (TCR) recognizes the antigen that is presented in the major histocompatibility complex (MHC-I or MHC-II) on the surface of the antigen presenting cells. This is called signal one. In the original activation model, the second signal needed to fulfill T cell activation implied the binding of CD28 to its B7 ligands, (CD80 and CD86). In the absence of this second costimulatory signal, no activation would take place.

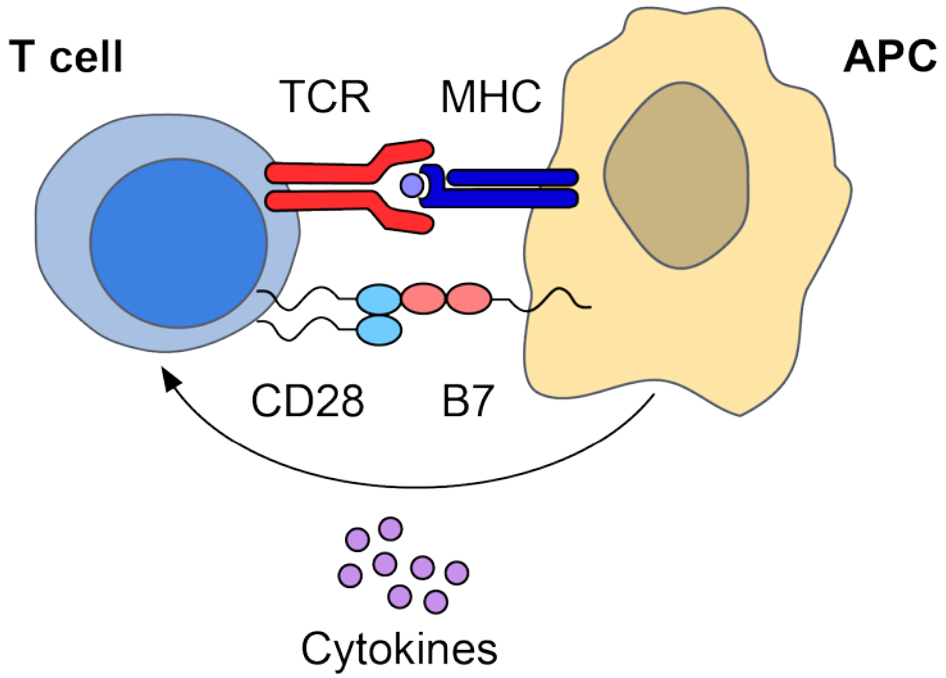

Figure 3. T cell activation is a multistep process. Signal one being the recognition of the TCR of the antigen presented in the MHC complex. The next signal, two is the co-stimulatory interaction where CD28 binds to one of its ligands. Both signal one and two are needed in order for activation to take place.

It is now known that T cell activation is fine-tuned with great precision using a series of co-stimulatory and co-inhibitory pathways that act together in order to deliver the precise amount of activation for T cells, enabling them to carry out their effector functions whilst preventing excessive immune responses. CD28 is the dominant co-stimulatory receptor on naïve T cells and other cells have other co-stimulatory receptors that are structurally related CD28, for instance ICOS (Inducible T cell co-stimulator) expressed by memory and effector T cells. Since activation cannot take place unless both signals are delivered, the receptors involved in the second signal act as molecular checkpoints for the activation of the immune response. These immune checkpoints are one of the main actors of peripheral tolerance, the mechanism which prevents the T cells that have escaped selection in the central tolerance from attacking self-antigen bearing cells.

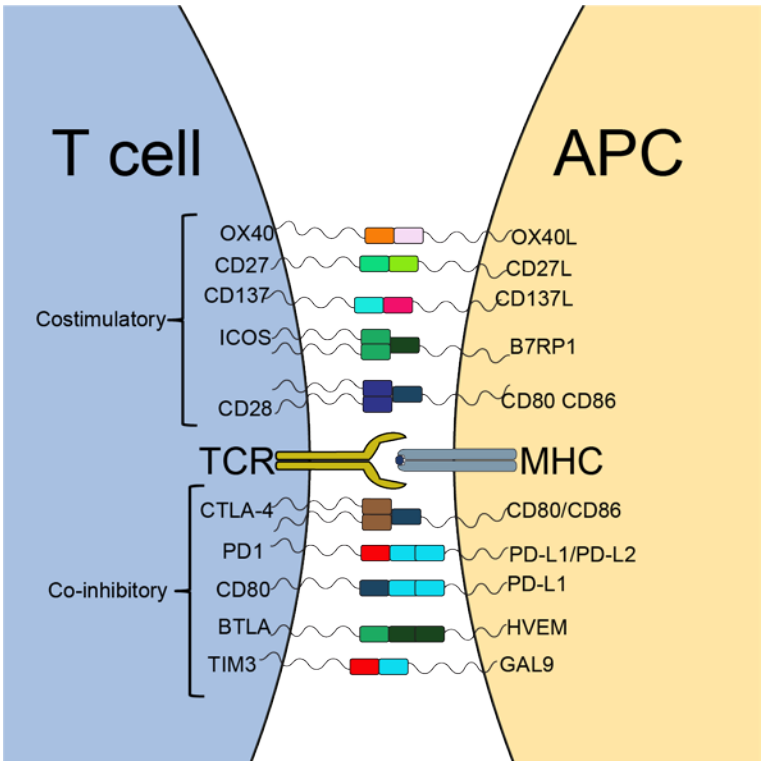

Figure 4. Different co-stimulatory and co-inhibitory pathways.
Pico de Coana et al, Trends in Molecular Medicine, 2015, Vol. 21, Issue 8

1.2.2.3 T cell differentiation

Once activated T cells will be ready to carry out their effector functions, which rely on the T cell subtype. Some subtypes of relevance to this thesis are:

T helper cells with the surface expression of CD4, provide help and assistance to other cells and are involved in the activation of cytotoxic T cells. They activate or inactivate other types of cells by secreting cytokines. Activated T helper cells can differentiate into at least five different subpopulations and each of these subtypes is characterized by a certain set of effector cytokines [50, 51]. The T helper cells of main importance for anti-tumor immunity are Th1 and Th2. Th1 secrete the cytokines interferon gamma (IFN-γ) and tumor necrosis factor –alfa (TNF-α). They are involved in producing an anti-inflammatory response and they could activate macrophages and are involved in the activation of cytotoxic T cells as part of the defense against intracellular bacteria, viruses and tumors. Th2 secrete the cytokines interleukin 4, 5 and 13 (IL-4, IL5, IL-13). They are mostly involved in aiding the differentiation and antibody production by B cells [59].

Cytotoxic T cells **(CTL)** with the surface expression of CD8 have, after being activated, the ability to recognize and kill cells that are infected or transformed. These cells present foreign (or transformed) antigens on their surface via MHC class I. CTL kill by establishing an immunological synapse with the foreign target and deliver signals that cause apoptosis, “the kiss of death”. They can kill in two ways, one faster way and one slower. They can either utilize the faster way by emptying the contents of cytotoxic granules with the molecules perforin and granzyme B into the foreign cell, which initiates a cascade of events finally inducing apoptosis. They can also kill trough death receptor-mediated killing, the slower way with the ligand FasL. This way involves cross-linking of the cell surface death receptor Fas expressed on target cells to the FasL expressed on CTL. Cross-linked Fas/FasL rapidly induces assembly of intracellular steps in the cell finally leading to apoptosis [50, 51].

T memory cells. Tumor specific CD8+ T cells undergo differentiation to effector T cells in the lymph nodes, proliferate and undergo clonal expansion and migrate to the tumor micro environment (TME) where they perform their action in killing tumor cells displaying tumor associated antigen expressed on the surface. But for long-term immunologic memory a part of the effector T cells have to develop into effector memory T cells [60].These cells have the capacity to rapidly re-expand to a large number of effector T cells if they are re-exposed to the earlier antigen. This gives the immune system a memory of earlier foreign cells. Memory T cells can be subtyped into central memory T cells (TCM cells) and effector memory T cells (TEM cells). T memory cells could be either CD4+ or CD8+ [50, 51].

Regulatory T cells **(Tregs**) are a subtype of CD4+ T cells and they have a major role in protecting the individual from autoimmunity by negatively modulating T cell responses. There are two types of Tregs: natural Tregs, which develop in the thymus and induced Tregs, which are derived from naïve CD4+ T cells, which have become activated in the presence of

TGF-β (Transforming growth factor beta) [61].TGF- β induces expression of FoxP3, an important transcriptional regulator responsible for the development and function of regulatory T cells. They negatively regulate the T cell responses either by secreting cytokines IL-10 or TGF-β which then inhibit the ability of antigen presenting cells to stimulate T cells, or they act more directly on T cells causing apoptosis. Tregs have also been demonstrated to negatively affect antitumor immunity both in mouse and in human studies [50, 51].

1.2.2.4 Other cells of importance involved in antitumor immunity of relevance to this thesis

MDSC, Myeloid Derived Suppressor Cells are a heterogeneous group of cells that have their origin in the myeloid lineage. They were first identified in the middle 1980s as "natural suppressor" cells in tumor-free mice where they inhibited T cell proliferation and cytotoxic T lymphocytes. In mouse models they are found as myeloid cells expressing high levels of CD11b (a myeloid lineage marker) and Gr1 (granulocytic marker). In humans there is extensive heterogeneity regarding the expression and level of cell surface markers. The phenotype of human MDSC is not as clearly defined as the one in mice, although they can be categorized as either monocytic or polymorphonuclear, based on their expression on CD14 versus CD15. They proliferate during pathological conditions such as chronic infection and cancer. They have strong immune suppressive ability especially on CD8+ T cells. They perform their suppressive activity through several mechanisms, primarily through the metabolism of L-arginine (an amino acid essential for the formation of the T cell receptor and thereby activation of the T cell). MDSC produce Arg1 (arginase-1) which degrades L-arginine thereby suppressing the T cell activation. MDSC also release ROS (Reactive Oxygen Species) that block T cell activation. They stimulate inducible NO synthase (iNOS) leading to the production of NO resulting in cell toxicity. In addition to all this they act through activation of IDO (Indoleamine-pyrrole 2,3-dioxygenase) resulting in tryptophan depletion leading to impairment of T cell growth and survival, and through upregulation of PD-L1 expression. In addition to direct T cell inhibition MDSCs can also induce and recruit T regulatory cells via TGF-β and IL-10 production and CD40-CD40L signaling [62-64] [50, 51]. In cancer patients it has been demonstrated that levels of circulating MDSCs correlate with clinical cancer stage and metastatic burden and that they are indicator of tumor progression [51].

NK cells are key players in the antitumor response (especially the innate) and were discovered in 1975 by Kiessling and coworkers [65, 66]. They originate from the same lymphoid progenitor as B and T cells and can be identified as CD 56+ and CD3- [67]. They can provide a fast response to virus infected cells as well as respond to tumor formation and they have the unique ability to recognize and kill stressed cells in the absence of antibodies and MHC I, which contributes to their rapid response. This ability is also behind how they were named, "natural killers" hinting that they do not need activation in order to kill cells that are "missing" self, MHC I [68]. There are two main subsets of NK cells; CD56 bright and CD56 dim. CD56 bright NK cells make up the majority of NK cells and are found primarily

in the bone marrow, secondary lymphoid tissue, liver, and skin and act by releasing cytokines [67, 69]. CD56 dim NK cells are found primarily in blood and are CD16 positive, the key mediator of ADCC (antibody-dependent cellular cytotoxicity), which characterizes a main mechanism how CD56 dim NK cells kill target cells [67, 69]. NK cells perform their effect through several mechanisms: cytolytic granule mediated apoptosis, ADCC, activation of CTL's, death receptor ligand, interferon gamma to mention some functions [50, 51]. Earlier it was thought that NK cells were involved mostly in the innate immune response, but it has become clearer that they as well are involved in the adaptive immune response, which make their role in anti-tumor immunity and in cancer therapies more important (ex. PMID: 29254979).

1.2.3 Immune response to cancer

The major role of the immune system is to protect us from invading pathogens, but when cancer develops, the pathogen is from within. Cancer cells are very similar to normal properly functioning cells so that under normal circumstances they should not be efficiently recognized and destroyed by the immune system due to the central tolerance of T cells. The transformed cells of tumors express antigens that are not present on normal cells. To the immune system, these antigens appear foreign, and their presence causes immune cells to attack the transformed tumor cells. The antigens expressed by tumors have several sources [70]. Tumor antigens are either TSA (Tumor-specific antigen) or TAA (Tumor-associated antigen). Tumor-specific antigens are antigens that only occur in tumor cells [71]. Mutation of protooncogenes and tumor suppressors which lead to abnormal protein production are drivers of the cause of tumor development and thus those abnormal proteins are the tumor-specific antigens, for instance abnormal products of *Ras* and *TP53* genes. Mutation of other genes unrelated to tumor formation may lead to synthesis of abnormal proteins that are called tumor-associated antigens. Tumor-associated antigens are present in healthy cells, but also occur in tumor cells and could then be used as tumor markers regarding some tumor types [71]. Melanoma is a tumor with a very high mutation rate, increasing the likelihood of generation of TAAs. There are certain types of specific melanoma TAAs. They were discovered by co-culturing lymphocytes with irradiated tumor cells, identifying the cytotoxic T cells that killed tumor cells bearing those TAAs. These melanoma-specific T cells can be found circulating in peripheral blood [54]. One of the most common melanoma TAA is MART-1 or MelanA, a melanocyte differentiation antigen that is a transmembrane protein present in melanocytes of normal skin, retina, nevi, and most melanomas [72-74]. Another known melanoma antigen is the cancer-testis antigen NY-ESO-1 [75]. Normal NY-ESO-1 expression occurs only in testis but its function is still mostly unknown. In melanoma it has been found in 20% of invasive tumors and has been associated with increased thickness of the primary tumor [75]. Other well-known melanoma TAAs are: MAGE-A3 (Melanoma-associated antigen 3) a member of the melanoma associated antigen gene family and its normal function in healthy cells is unknown. Gp100 (glycoprotein 100 or melanocyte protein), a transmembrane protein enriched in melanosomes, the melanin producing organelles in the melanocytes, which is involved in melanosome maturation [76].

When CTLs encounter a cancer cell presenting the matching tumor antigen, the CTLs can lyse the cancer cell, resulting in the release of more TAAs and continued loop of destruction of the tumor.
When T cells infiltrate the tumor they are known as tumor infiltrating lymphocytes or TILs. These tumor specific T cells are also found circulating around in the body and along with TILs, their numbers can be used as a prognostic marker for cancer treatment with immunotherapy where the immune system is utilized to eradicate cancer [77].

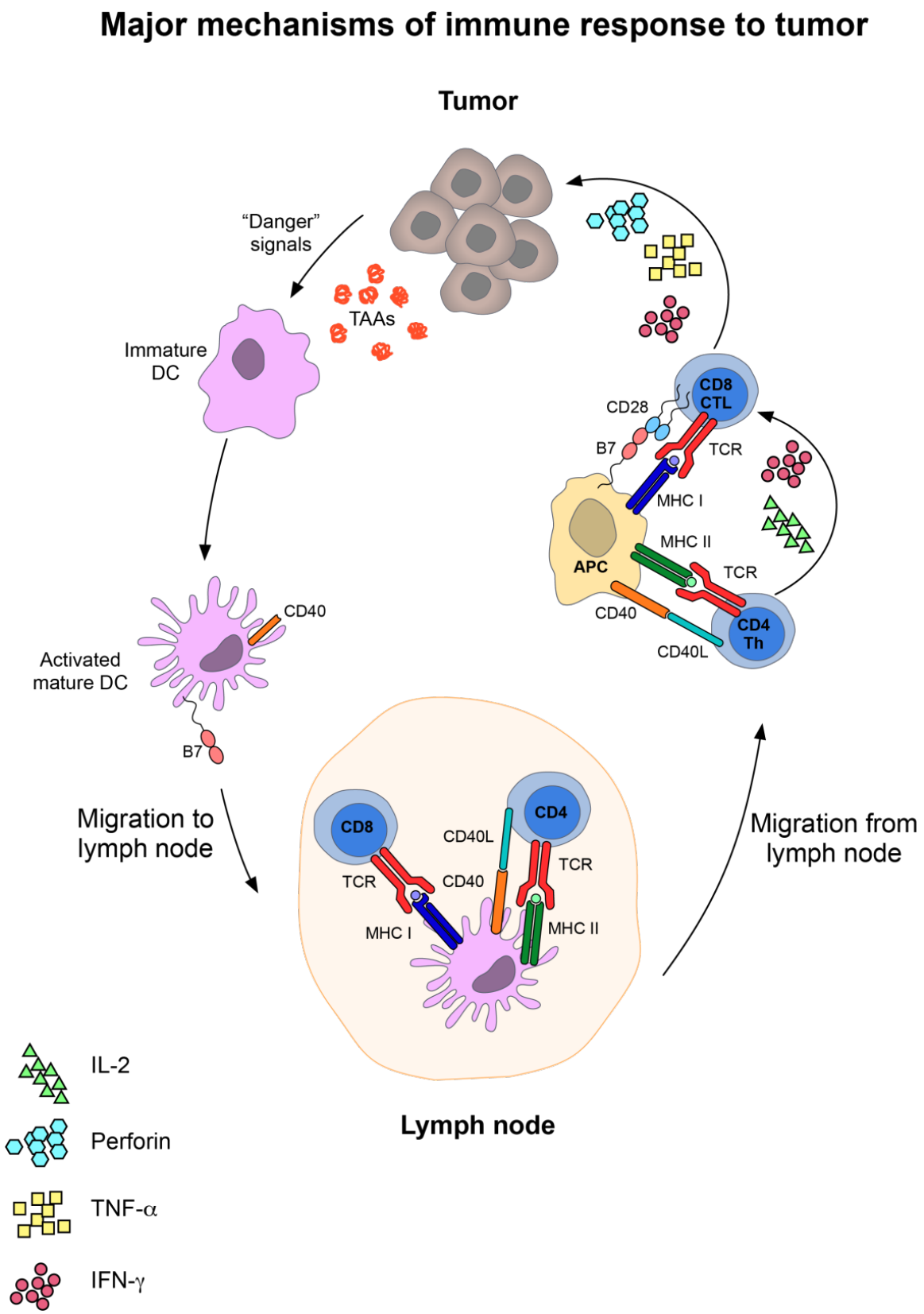

Figure 5. Immune response to cancer: Tumor formation gives rise to antigens (TAA). These are recognized by antigen presenting cells. The antigen presenting cells migrate to the lymph nodes where, priming and activation of T cells take place, i.e. naïve T cells become activated. After activation, T cells traffic to the periphery and the tumor site, where the T cells perform their action, eradication of tumor with several modalities.

1.2.4 Immunoediting

One important role of the immune system is to identify and eliminate tumors. When cancer cells are attacked by immune cells the immunogenicity of tumors can change through mutations and selection of immune-resistant cancer cells.
The relation between the tumor cells and the immune system is a dynamic process known as immunoediting. It is made up of three phases: elimination, equilibrium, and escape, “three E’s of cancer immunoediting” [78]. Simplistically, tumors proceed through each of these phases, but in reality it is more likely that transitions between the phases occur in both directions.

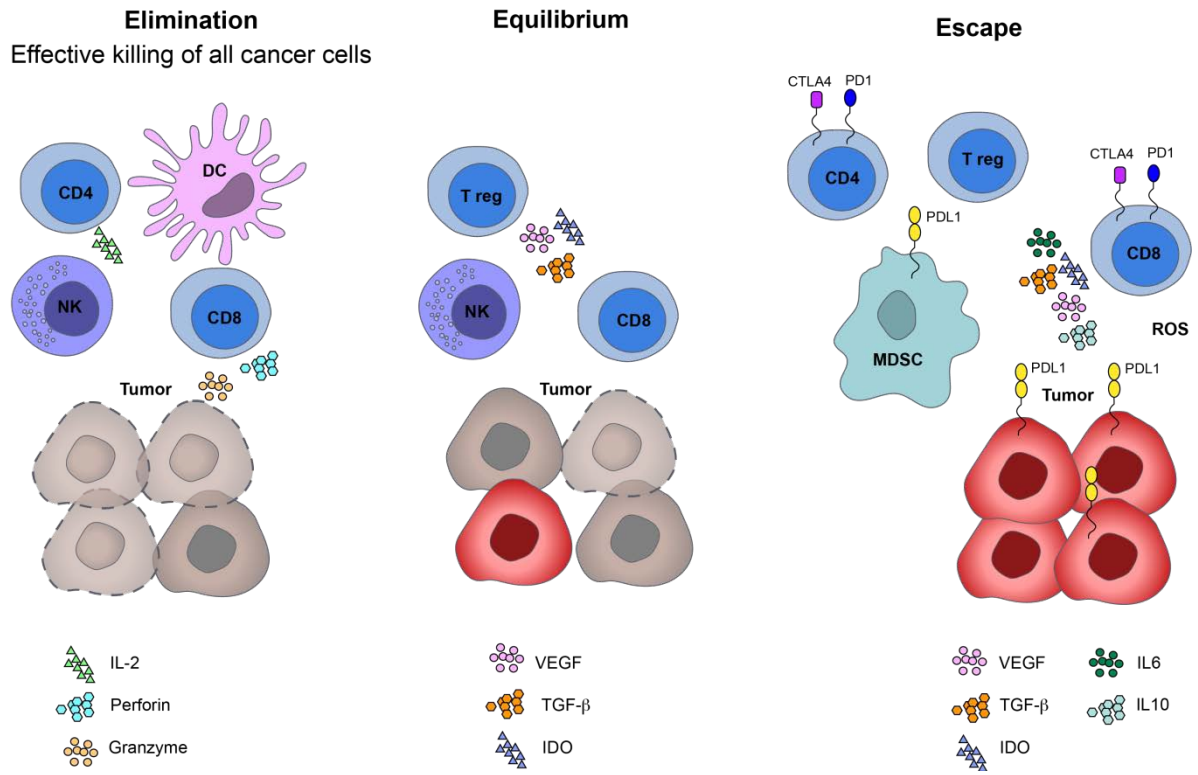

Figure 6. Immunoediting: In the elimination phase the immune system, both the innate and the adaptive, recognizes and eliminates the tumor cells. During equilibrium phase tumor cells survive the elimination and co-exist with the host during strict control with different immune defenses. During escape the tumor have got the ability to circumvent immune responses and overcome immunity with different mechanisms.

The **elimination** phase, also known as immunosurveillance, is the initial phase, in which the immune system is able to identify transformed cells and successfully eliminate them.

During the **equilibrium** phase, tumor cells through mutagenesis can acquire features increasing their resistance to elimination by the immune system. Most of the transformed cells are eliminated by the innate and adaptive immune response, but it is no longer possible to eradicate them completely.

At a certain point, tumor cells are no longer eliminated by the immune system, they overcome it and manage to completely **escape** it. There are several mechanisms that lead to escape of cancer cells from the immune system [79], including downregulation or loss of expression of classical MHC class I [80], defects in antigen processing, development of a tumor microenvironment (TME) with suppressive effect on the immune system [81]. Cells in the tumor microenvironment are able to produce cytokines and other mediators which can cause apoptosis of activated T lymphocytes [82]. Among the cells that increase in the TME during cancer growth are MDSCs, which are found to be increasing in patients with metastasized melanoma [83, 84] and they, as mentioned earlier, suppress both CD4+ and CD8+ T cells and inhibit T cell production of interferon-gamma (IFN- γ, a very important cytokine involved in immune regulation and anti-tumor response). They also facilitate the development of other cells that promote tumor progression (macrophages, T regulatory cells). It was also shown, by our group, that the frequency of a subgroup of MDSCs (MM-MDSCs; CD14+/HLA-DR -/low) in circulation return to physiological if the patient did not have active disease or was in regression, indicating a causal correlation between disease status and the presence of MDSCs [85]. It has also been shown that the frequency of circulating MDSCs in peripheral blood correlate with tumor progression and worse outcome in patients with different tumors [86-90].

1.2.5 Immunogenic cancer

Malignant melanoma is regarded as the prototype for immunogenic cancer, which means that it is affected by and affects the immune system, and this is based on several observations. Spontaneous tumor regression was observed already in the 1950s [4] and can be seen both in primary melanoma and in advanced disease where it is rare though [91]. The importance of TILs (tumor infiltrating lymphocytes) was first described by Clemente et al, showing that different infiltration grade of TILs in melanoma was related with survival [92] [93], which was also supported by later observations [94][95]. When TILs are enriched and activated they are capable of rejecting tumors [96], which is the main principle behind our therapy described later in this thesis. There is an increase in the incidence of melanoma in patients receiving solid organ transplantation as a result of the immune system being suppressed [97] [98]. Melanoma is one of the malignancies with the largest number of mutations [99], leading to many tumor antigens being presented, which in turn activates the immune system greatly, and is thought to be one of the reasons behind its immunogenicity. Finally, the cancer is exceptionally responsive to and affected by immunotherapy.

1.3 SYSTEMIC TREATMENTS OF ADVANCED DISEASE

In the beginning of the 2000s my relative started to have some symptoms. A pain from one side of the thorax appeared which was thought to be related to physical exercise. When the pain did not improve x-ray was performed and revealed something in one of the lungs, which lead to follow-up, though it was thought that it might be sarcoidosis. Coupled with the pain was a tiredness which was explained with having too much work. After almost half a year and another couple of x-rays the pulmonary lesion was biopsied and revealed melanoma. By then he had deteriorated a lot, experiencing pain and not being able to work. Then it all went very fast. After diagnosis of spread melanoma he died in a few months, never making it to treatment, which at that time was chemotherapy. This was only around 20 years ago and in that period so much has happened to the treatment of advanced disease.

1.3.1 Historical/earlier systemic treatments of advanced disease

1.3.1.1 Chemotherapy

The systemic treatments available in the beginning of the 2000s were limited to chemotherapy, such as dacarbazine (which had been approved in the middle of the 1970s [100]) and its oral analog temozolomide. For dacarbazine ORR of up to 20% has been reported but CRs are rare (about 3–4%), and duration of response is short (median 5–6 months), with only a few percent of patients experiencing long-term survival [101, 102]. Temozolomide has the ability to penetrate the blood brain barrier and demonstrated modest antitumor activity [100, 103]. A randomized clinical phase III trial with treatment naïve metastatic melanoma patients showed similar median OS for patients treated with dacarbazine and temozolomide [104], leading to more use of the oral drug in the clinical setting in Sweden. Other treatments were available internationally, most of them being immunotherapy and most of them in clinical trials.

1.3.1.2 Immunotherapy

History

Immunotherapy is the "treatment of disease by inducing, enhancing, or suppressing an immune response" and cancer immunotherapy is the use of the immune system to treat cancer. Immunotherapy began in 1796 when Edward Jenner produced the first vaccine involving immunization with cowpox to prevent smallpox [105]. William Coley (1866-1936), although not recognized during his lifetime, has thereafter been described and recognized as "the father of cancer immunotherapy". Coley found that patients with cancer sometimes developed durable regressions after having an infectious disease [106]. This he further investigated by injecting cancer patients with mixtures of live and inactivated Streptococcus pyogenes and Serratia marcescens, 'Coley toxins'. With this method he achieved some responses including durable ones, but with the risk of severe infections. The lack of a known mechanism of action for 'Coley's toxins' and the risks of deliberately infecting cancer patients with pathogenic bacteria contributed to establishing other treatments

against cancer [107]. By end of the 19th century Emil von Behring and Shibasaburō Kitasato discovered that injecting animals with diphtheria toxin produced blood serum with antitoxins to it [108]. In the beginning of the 20th century Paul Ehrlich's (1854-1915) research gave rise to the "magic bullet" concept; using antibodies to specifically target a disease. The idea of using immunotherapy in cancer, in general, received recognition when Thomas and Burnet first proposed the theory of cancer immunosurveillance in 1957 [109]. The strategy of using attenuated bacteria to treat malignancies reappeared in 1976 when a trial was conducted to test the use of the tuberculosis vaccine Bacille Calmette-Guérin (BCG) to prevent the recurrence of non-muscle invasive bladder cancer [110]. BCG therapy was shown to be very effective and continues to be used today. The cytokine interleukin-2 (IL-2) was identified and purified in 1976 allowing investigators to culture T cells in vitro for the first time [111]. The use of IL-2 as an immunotherapeutic drug gained US Food and Drug Administration (FDA) approval for metastatic kidney cancer in 1991 and for metastatic melanoma in 1998. In the beginning of the 1970s the invention by Jerne, Köhler and Milstein of a method to produce monoclonal antibodies laid the ground for antibody therapy in the clinic, and was awarded a Nobel Prize in 1984. In the beginning of the 1990s the use of vaccines in the treatment of malignancies was developed further, but effective vaccines remained elusive. Thereafter the development of melanoma immunotherapies is a success, which will be described further below.

Cytokines

The connection between melanoma and the immune defense had been considered for some time but the benefit of treating melanoma with immune therapy was first realized in the 1980s when the cytokines interleukin-2 (IL-2) and interferon-alfa (IFN-α) became available in large quantities [112, 113]. Phase II trials with high dose IL-2 showed durable remissions in 16% of patients with advanced melanoma and a median duration of response of 8.9 months and as long as almost 60 months in the 6% of the patients who were complete responders [114][31]. Evidence of long term disease control led to approval of IL-2 by FDA in the 1990s. However, the treatment is sometimes associated with severe toxicity such as hemodynamic collapse and therefore, along with the fact that no survival benefit has been proven, the use of IL-2 for the treatment of advanced melanoma has remained restricted and IL-2 is not yet approved as a drug in Sweden. IFN-α also has some measurable effect in melanoma and in small phase I/II trials in the 1970’s and 1980’s it’s limited response rate was predominantly seen in patients with limited disease burden [113]. Subsequent trials focused on its use in the adjuvant setting.

The benefit from the treatments with cytokines (IL-2 and IFN-α) was limited and associated with toxicity and therefore their role in the clinical setting has been limited. The toxicity of these treatments is caused by their mechanism of action, leading to a non-specific stimulation and upregulation of the immune system.

Cancer vaccines

Other earlier developed treatments available by the beginning of this century aimed at generating a more specific T cell response as tumor vaccination and adoptive cell transfer (ACT).

Vaccination is a preparation of microorganisms administered in order to produce or artificially increase immunity to a particular disease. Vaccination can be categorized as prophylactic or therapeutic. One of the first recorded instances of vaccination is from China circa 1000AD and was made by variolation. This method later spread to Europe and was pioneered by Edward Jenner (1749-1823), see above. The idea of vaccination against cancer was initiated by Paul Ehrlich (1854-1915), who was the first to propose an immune-tumor interaction. He attempted to use weakened tumor cells for immunization in cancer patients, but this was no success. William Coley (1866-1936) on the other hand had more but dual success with the method of using bacteria to treat malignancies, see above. Further details regarding the history of cancer vaccines are described above. In the 1950s the development of antitumor vaccines saw the real light when the presence of cancer specific antigens potentially inducing an immune response was first observed [115]. Thereafter many clinical trials of therapeutic vaccination have focused on melanoma, with the results being mostly disappointing and not able to show improved overall survival of melanoma patients, despite some promising data in animal models and a few early trials.

However, in combination with other treatments vaccination has been shown to be somewhat more effective. For instance gp100 and HD (high dose) IL-2 that in a randomised phase III trial showed benefit of the combination arm of gp100+HD-IL 2 vs HD IL-2 alone both regarding response rate and OS [116].

Adoptive cell therapy

ACT has been proven to induce impressive along with durable responses in patients with advanced melanoma. ACT was developed in the late 1980s by Rosenberg and colleagues by using the basic technique of purifying TILs from a patient's fresh tumor, expanding the cells in culture ex vivo, and re-infusing them along with a short course of IL-2 [96, 117].

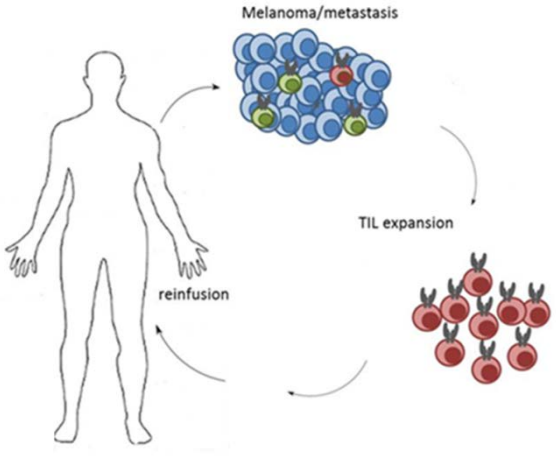

Figure 7. Adoptive cell therapy, ACT (L. Rehn, 2017)

Early trials reported objective responses in more than 30% of the treated patients [96] [118]. Thereafter multiple single-institution studies in patients with metastatic melanoma have demonstrated response rates that reach 50% with ACT, as well as complete response rates in about 20% of patients, most of which have been durable CRs [119] [120]. Despite the very good response rates and durable responses the treatment is not an approved treatment for patients with stage IV melanoma, but rather a treatment for a well selected group of patients. This treatment method will be described further and reflected upon later in this thesis.

So by the beginning of this century there were some different treatments available to offer patients with MM but none of the approved ones being able to result in prolonged OS.

1.3.2 The new revolutionizing treatments of advanced disease

There were, until less than a decade ago, only few treatment strategies to offer patients with stage IV melanoma and the median overall survival, as mentioned above was very short. The treatments were limited to palliative surgery, radiotherapy and palliative chemotherapy. I recall there was sort of a "hunt" for removing stage IV metastases with surgery or targeted radiation if possible. This was because there was a belief that perhaps by removing the metastasis not only the symptoms would improve but the survival as well [49] [121], but that was debated.

When I started working at the melanoma unit at Karolinska University Hospital in 2010 I remember the clinical trials for advanced melanoma going on by then; one trial with anti-CTLA-4, ipilimumab and one with the BRAF inhibitor vemurafenib. I remember the difficulties I had understanding and differentiating between the different mechanisms of action and I guess I was not the only one. However it was fascinating to have something to actually offer the patients and a feeling that something big was happening. Since then, starting 2011 with the first approval among these new treatments, so much has happened.

1.3.2.1 Immune Checkpoint Inhibitors; anti-CTLA-4

In the end of the 1980s, researchers identified cytotoxic T-lymphocyte antigen 4, or CTLA-4, a member of the immunoglobulin superfamily and a transmembrane receptor which is expressed on activated T lymphocytes [122-124]. It is one of two homologous cell surface proteins that counterbalance each other in the activation and inhibition of T cells.
CTLA-4 has a stronger affinity to its ligands CD80 and CD86 on antigen presenting cells than its counterpart CD28. CD28 transmits a stimulatory signal [125] whereas CTLA-4 transmits an inhibitory signal [126-128] to T cells. Binding of CTLA-4 to CD80 and CD86 leads to down-regulation of T-cell activation by induction of T-cell anergy and inhibition of IL-2 secretion. This is a protection mechanism, a check-point, preventing the activation of T cells from becoming excessive and leading to attack on the organism itself. CTLA4-deficient mice suffer from fatal lymphoproliferative disease characterized by multi-organ T cell infiltration and die by 3–4 weeks of age, indicating that CTLA4 is an essential negative regulator of T cell responses [123, 124].

Allison found that CTLA-4 prevents T cells from attacking tumor cells. In 1996, he also showed that antibodies against CTLA-4 allowed the immune system to destroy tumors in mice [129]. These preclinical findings led to the clinical development of two humanized anti-CTLA-4 monoclonal antibodies: ipilimumab and tremelimumab. Eventually also leading to the Nobel Prize in medicine in 2018.

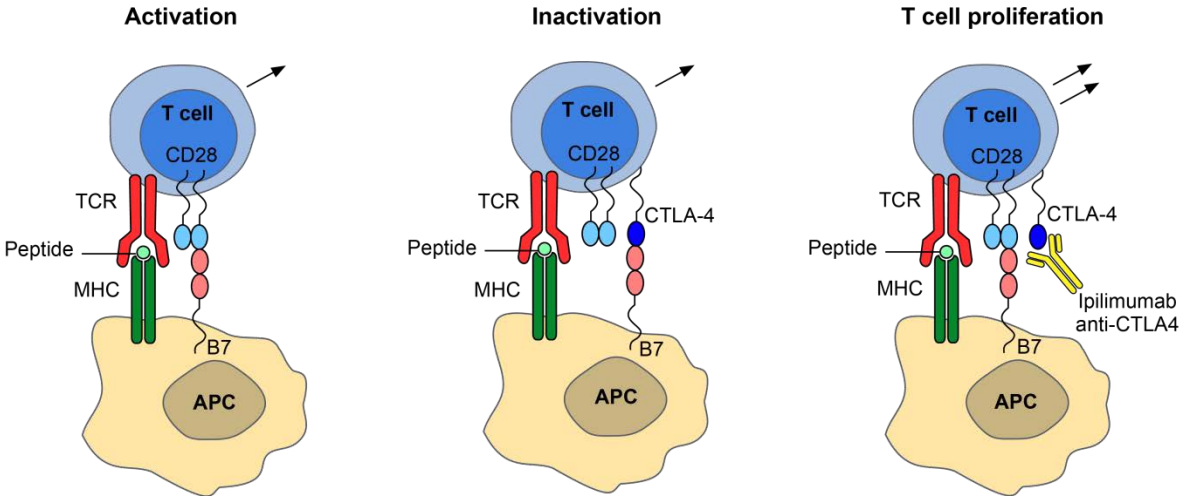

Figure 8. CTLA-4 and CTLA-4 antibodies: When T cell activation takes place CTLA-4 gets activated and acts as a break, in order to protect the individual from too excessive activation of T cells and thereby autoimmune reactions. CTLA-4 has a greater affinity for B7 than CD28, which makes this protection mechanism workable. Anti-CTLA-4 antibodies block CTLA-4 and thereby "block" the break and consequently activate the T cell response.

In early phase clinical trials both agents demonstrated an ability to induce durable clinical responses in small subsets of patients with advanced melanoma [130-132]. Ipilimumab was thereafter evaluated in two revolutionizing clinical trials published in 2010 [133] and 2011 [134]. Ipilimumab was the first drug ever to show improved overall survival for metastatic melanoma and this lead to FDA approval in 2011 for second line treatment and in 2013 for first line treatment. Despite the revolutionizing improvement of OS, only a limited number of patients, less than 20%, respond to treatment with ipilimumab, but long-term survival data indicates that 20% of patients show evidence of continued durable disease control or response 5-10 years after starting therapy [135].

There is no pretreatment predictive marker of response or long term survival. The response to the treatment may also occur late, sometimes up to more than 6 months after the end of the treatment schedule and a late response can be preceded by an early progression (often in the first 6-12 weeks of treatment), so called pseudo-progression, of the disease, which was reported to occur in about 10% of treated patients [136, 137]. This together makes the treatment rather difficult to handle in a real life clinical setting and not suitable for all patients with generalized melanoma, especially when used as second or third line treatment. The ideal patient for treatment with ipilimumab has good performance status, limited slow progressing

disease and no lesions threatening vital organs and the patient should be able to tolerate a pseudo-progression.

The treatment-related specific toxicities are of autoimmune kind and they are frequent. More than 70% of the treated patients will experience side-effects which may be severe and in some cases potentially fatal if not discovered in time and treated with high-dose cortisone. One of the most common toxicities with ipilimumab treatment is colitis which usually occurs during the first 1-2 months of treatment and can rather easily be managed if recognized in time. The more unusual toxicities of endocrine and neurological kind more often occur later after the treatment initiation and may be more difficult to treat.

Taken together, the fact that only few patients respond to the treatment, the difficulty to select the right patients and the frequent and sometimes severe side-effects make the treatment, although it being revolutionizing in its kind, difficult to use and monitor in the clinical setting.

1.3.2.2 Targeted therapy, inhibitors of the MAP kinase pathway

Another group of systemic treatment, oral kinase inhibitors was in many ways much easier to manage in the real life clinical setting and the first of these drugs was also approved in 2011. A precondition for this type of treatment to function is that the tumor harbors a *BRAF*V600 mutation, which is the case in about 40-60% of cutaneous melanomas. This mutation causes hyper activation of the MAPK pathway leading to increased cell division and tumor growth.

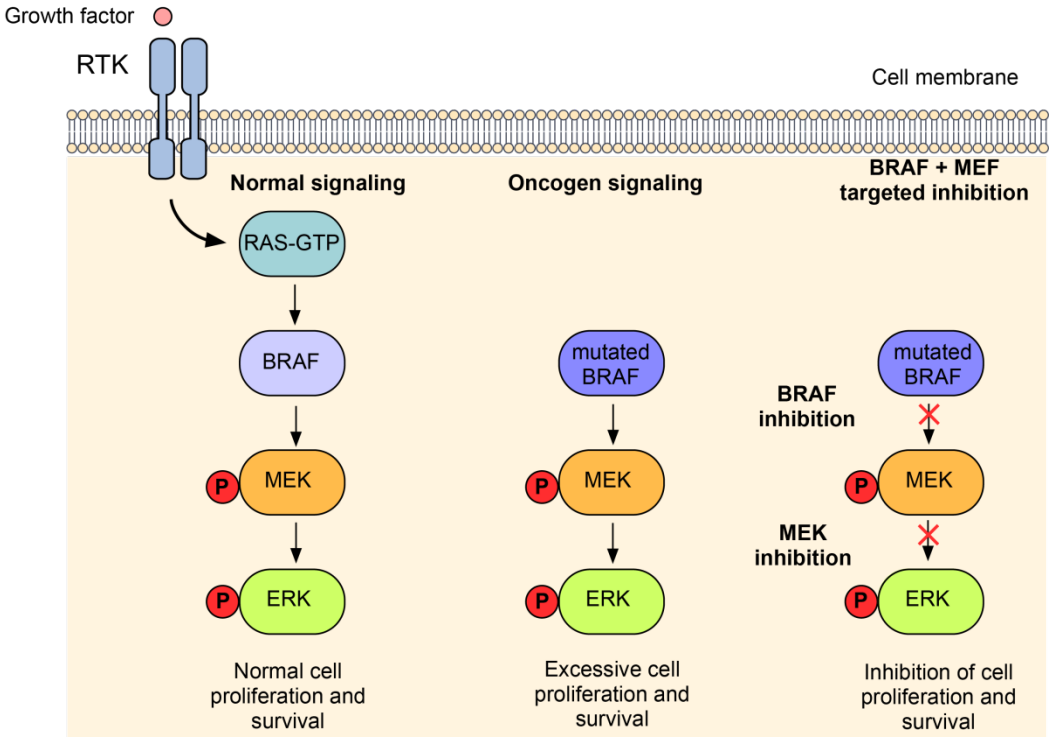

Figure 9. MAPK pathway: Normal activation of the pathway occurs when extracellular growth factors bind to the receptor, then resulting in downstream activation of the pathway, eventually leading to normal cell proliferation and survival. Mutations of *BRAF* result in constitutive activation of the pathway without any upstream activation through growth factors. This causes excessive cell proliferation and survival. With the use of BRAF and MEK inhibitors, the activation gets halted in several steps in the excessive down-stream activation.

The approval of the first BRAF inhibitor, vemurafenib was based on a phase II trial [138] showing that overall response rate for patients with advanced melanoma was more than 50% and a phase III trial comparing vemurafenib with dacarbazine, showing much better response rate (50% vs 5%) and both improved progression free survival and overall survival for the vemurafenib cohort [139, 140]. After that another BRAF inhibitor, dabrafenib was approved, showing a similar effect in a phase III trial [141], but with different and more manageable toxicity profile.

When first using BRAF inhibitors it was very compelling to be able to offer them to patients since the response rate was high, the symptoms could improve already after a short time and the performance status of the patients as well could improve. From being severely ill from a deadly disease, the patients could become almost free of symptoms and sort of get his or her life back, which was also very satisfying for us clinicians being a bit like magicians. Unfortunately, then comes disease progression, due to acquired resistance through reactivation of the MAPK pathway [142, 143].With BRAF inhibitors alone it came already after about 6-8 months and often it came very rapidly and the progression was frequently stormy. The hope that slowly had been built up was harshly destroyed again. One could really say that there was a shadow around the initial response, since you sort of knew that the progression eventually would come. That, coupled with the not so pleasant toxicity of the single drug use of BRAF inhibitors, especially vemurafenib (rash, photo-sensitivity, arthralgias, hair-loss, and squamous cell carcinoma (SCC)-like lesions) [139] made the clinical use of single agents somewhat dubious. The situation became better when the combination of BRAF and MEK inhibitors was approved in 2015. The approval was based on several trials showing better response rates, better progression free survival, better overall survival and improved toxicity profile compared to treatment with BRAF inhibitor alone [144-147]. Using this combination in the clinical setting was easier, since the toxicity was milder, the duration of response more satisfying and when progression came, perhaps it seemed like it did not appear as rapidly as with single use. Although patients in most need for the rapid response of the targeted therapy; those with large tumor burden, many sites of metastases, high LDH and symptoms from their disease, still progressed faster than patients with a more favorable situation, indicating that low disease burden at baseline can be prognostic for a long term benefit [148, 149].

Targeted therapy was more manageable in the clinical setting than anti-CTLA-4, but when another type of check-point blockade was introduced the arena changed again.

1.3.2.3 Immune Checkpoint Inhibitors; anti-PD-1

In the early 1990s, Tasuko Honjo identified a molecule expressed in dying T cells, which was named programmed death 1, or PD-1 and which was recognized as another regulator with negative effect on T cells. Programmed death-1 (PD-1) is a checkpoint receptor that also belongs to the CD28/CTLA4 receptor family [150] [151] [152]. PD-1 binds to two known ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) which are widely expressed in a variety of tissues [153, 154]. When PD-1 binds to PD-L1, it negatively regulates T cell functions [151]

[152] [153]. PD-L1 is expressed in many tumors, including melanoma [155, 156]. PD-1/PD-L1 interactions have been studied in animal models, as well as in vitro, and the complex has been shown to inhibit the effector functions of tumor-specific CD8+ T cells, thereby contributing to tumor-induced immunosuppression with tumor resistance to cytotoxic T cell responses [155-157].

Unlike CTLA-4, which is primarily involved in immune cell activation and generation of early responses, the PD-1 system is important in immune escape at the point of CTL mediated cell-killing, and seems primarily designed to limit overresponse to infection and prevent autoimmunity in the periphery [151, 158,159]. In contrast to the rapidly appearing systemic autoimmunity observed in CTLA-4-deficient mice, PD-1 deficiency results in delayed-onset, organ-specific autoimmunity. Depending on their genetic background these mice within some months develop autoimmune symptoms such as lupus-like syndromes and autoimmune cardiomyopathy syndromes [160-162]. This suggests that PD-1 might function more as an inhibitor of lymphocyte responses in the periphery. Chronic antigen exposure from viral antigens has been observed to induce PD-1 expression and create a state of anergy, or immune non-responsiveness in antigen-specific T-cells. A similar upregulation of PD-L1/L2 is observed on tumor cells [82]. The inhibition of T cell function through the activity of PD-1 is therefore a critical therapeutic target for the activation of T cells. This rationale has led to the development of monoclonal antibodies that block the activity of PD-1 receptors. These antibodies block the PD-1 receptors on T cells, making them unable to respond to PD-L1 or PD-L2 expressed on tumor cells. This, in turn, leads to the activation of T-cell activity and a decrease in tumor growth.

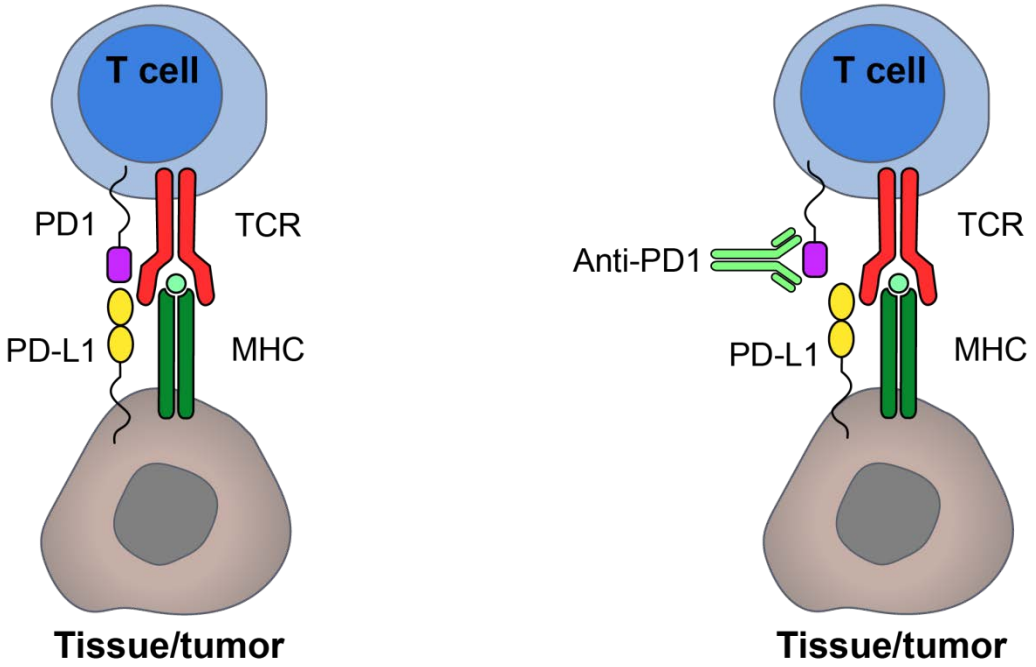

Figure 10. PD-1 check-point acting in the periphery and anti-PD-1 antibodies.

Soon after the development of ipilimumab, data from early trials describing the clinical activity of the anti-PD-1 antibodies emerged with response rates around 30% and with a much more favorable adverse event profile [163-165].

Pembrolizumab was the first anti-PD1 antibody to be approved by the FDA for the treatment of metastatic melanoma. Three trials led to the approval of pembrolizumab: the phase I trial Keynote-001, the phase II trial Keynote-002 and the phase III trial Keynote-006 [166-168]. It was initially approved for the treatment of metastatic melanoma after progression on ipilimumab.
The second anti-PD1 antibody to be approved was nivolumab and the approval was based on two trials, CheckMate-066 [169] and CheckMate-037 [170, 171] and as with pembrolizumab it was initially approved to be used in the setting of advanced melanoma progressing on ipilimumab.
The real effect on overall survival cannot be evaluated in the above mentioned trials as none of the trials was designed to evaluate that endpoint. Thus more long-term follow up has shown positive results regarding response rate, response duration and overall survival rate at both 3 years and 5 years for patients treated with pembrolizumab and nivolumab [168, 172-174]. Cross study comparisons of homogeneous groups of patients treated with pembrolizumab or nivolumab monotherapy have similar results regarding clinical endpoints and adverse event rates [175]. Their efficacy has been shown both for treatment naïve patients and previously treated patients, which the above mentioned trials suggest.

Anti-PD1 antibodies moved very fast from initially being used in the last line setting to become established as frontline therapy for metastatic melanoma, because of their higher response rates, more tolerable toxicity profile, and fewer grade III or IV adverse events (AEs) than ipilimumab. PD-1 inhibitors are overall better tolerated than ipilimumab, and even though fatigue is a common problem (16-24%), irAEs occur less frequently and are more often of lesser severity (<12% grade 3 or higher events) [176].The spectrum or organs involved is similar to ipilimumab, although there is a higher incidence of pneumonitis and thyroid dysfunction.

Despite the success in the clinical setting, with the much improved response and toxicity profile, there are a still only a limited number of patients that benefit from treatment, albeit they could be long-term responders or even cured and treatment could be stopped. With the toxicity profile being as mild, it is easier to be tempted to use these drugs even though the clinical parameters indicate that there is a risk of the patient not benefitting; large tumor burden, elevated LDH, but perhaps still rather good performance status. With ipilimumab it was in some sense easier to make the decision that clinical benefit most certainly would not happen, but with anti-PD1 it is easier “to try any way” which could be considered from both a good and a bad perspective.

1.3.2.4 Combination of Immune Checkpoint Inhibitors; anti-CTLA-4 and anti-PD-1

CTLA-4 and anti-PD-1 perform their action in different parts of the immune activation cascade. CTLA-4 is thought to affect the immune priming phase by regulating T-cell proliferation early, primarily in lymph nodes, whereas PD-1 suppresses T cells later during the effector phase, primarily in the peripheral tissues, or in the tumor bed during antitumor response [158].

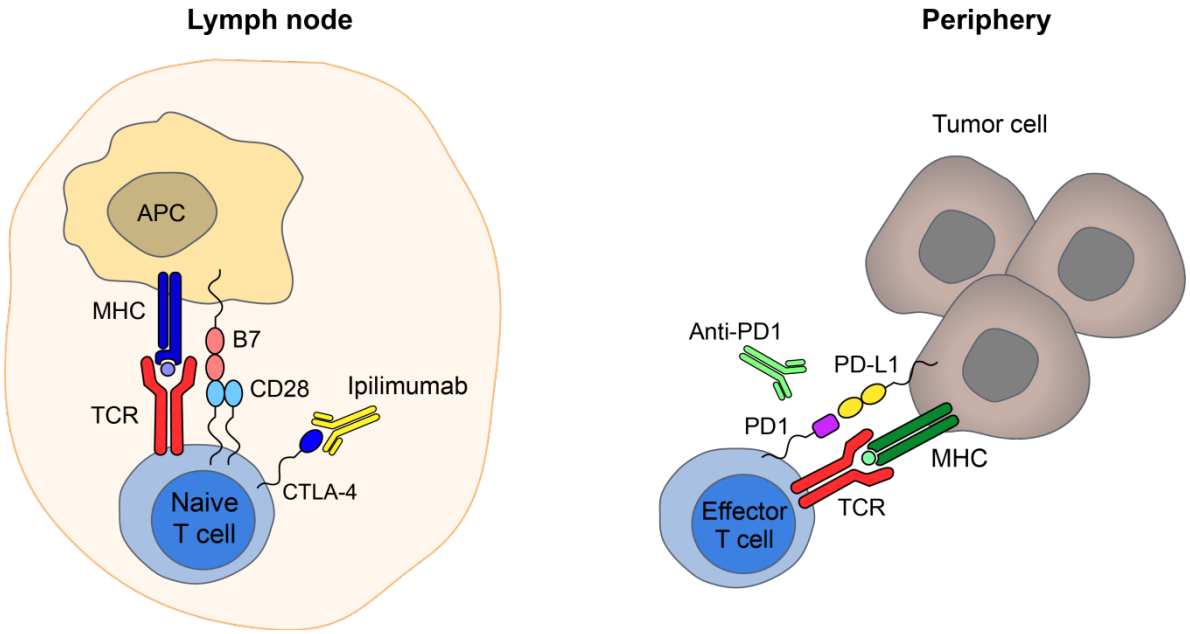

Figure 11. Dual treatment with both anti-CTLA-4 and anti-PD-1 antibodies.

This difference lead to the use of the combination of anti-CTLA-4 and anti-PD-1 in order to achieve better efficacy, which was also shown in the pivotal trial check-mate 067, where a much higher rate of response and better PFS was seen in patients treated with the combination [177, 178]. This trial also found that patients with tumors lacking PD-L1 expression had an overall survival benefit [177, 178]. The data have recently been updated regarding 5-year overall survival showing a slight, but not statistically significant better overall survival for the combination as compared to anti-PD-1 single use [179].
However, the better efficacy really comes with a high price for many of the patients with the toxicity being much higher for the combination compared to single use of both anti-CTLA-4 and anti-PD-1, with grade 3-4 toxicity in about 55% of patients in the combination arm in the check-mate 067 trial. Thirty-six percent of patients in the combination arm discontinued treatment, but among those, 70% maintained the achieved therapeutic response.
The most common AEs of any grade with the combination were gastrointestinal toxicity, asthenia, and pruritus, followed by rash, loss of appetite, nausea, and pyrexia [178].
The more severe toxicity can be treated with high dose corticosteroids or TNF-alfa inhibitors.

Despite that, severe toxicities may lead to durable hospitalizations and sequels which take very long to recover from and thereby decreased quality of life. The severe toxicity is of autoimmune type and can affect most organ systems. It seems though, form a pure clinical perspective, that the immune related toxicities sort of mimic an original autoimmune spectrum of disorders and that they are more difficult to diagnose and to treat than the original autoimmune disorders.
It is easy to be impressed by the very, very good response rates and not to think so much of the AE.
The treatment really is not a true savior and one must ask oneself if it is better to have decreasing tumors but suffer with sequels form toxicity that makes one's life very deprived of quality. It makes me think of "horror autotoxicus" and that Ehrlich did not think that the immune system could attack the organisms own tissue, which was later shown by his student Witebsky that it could [180]. It is my clinical feeling that can arise when meeting the patients suffering from severe complicated toxicity caused by the combination therapy, "horror autotoxicus".

This harsh clinical setting has resulted in reconsidering of combination immunotherapy and to trials where the dosage of ipilimumab and nivolumab is altered with higher dose of nivolumab at 3 mg/kg and a lower dose of ipilimumab at 1 mg/kg. For instance Keynote-029 showing preliminary results with high activity (PFS of 70% at 6 months), similar to that observed with the initial regimen, but with 25% immune-related grade 3-4 AEs [181]. Another trial, check-mate 511 with the dosage of nivolumab at 3 mg/kg and ipilimumab at 1 mg /kg showed correlative effect on treatment response, progression free-and total survival as with the initial dosing of nivolumab 1mg/kg and ipilimumab 3mg/kg, but significantly lower frequency of serious (grade 3-5) immune related toxicity [182]. This regimen has sought its way more and more into the clinical setting, which is an advantage for the patients and a relief to us prescribers. This combination has also been tried in another setting; in different treatment lines to more heavily treated patients with some good preliminary results [183].

The sequence of immune therapy has also been considered. Is it more favorable to treat with anti-CTLA-4 before anti-PD-1 or vice versa? Anti-CTLA-4 agents can upregulate PD-L1 expression, potentially enhancing the action of a subsequent PD1/PD-L1 inhibition in the tumor microenvironment [184]. Some patients with advanced disease are in the need of a rapid tumor reduction. CTLA-4 activation, as mentioned above, mediates an earlier phase in the immune response than PD-1. To induce an antitumor response, ipilimumab has to activate T cells in the lymph nodes. Thereafter these T cells could migrate to the tumor site, while anti-PD-1 antibodies can activate lymphocytes directly in tumor microenvironment. This has a clinical implication, as ipilimumab´s activity occurs slower than that of anti-PD-1 antibodies. Thus initial administration of anti-PD1 antibodies could lead to rapid responses, and sequential ipilimumab could result in enhanced antitumor activity. The efficacy of sequential treatment has, as mentioned above, been tried in different settings [185-189], but neither of them so far being able to offer a consensus for the clinical setting. There is quite some debate in the field regarding the most favorable way to combine anti-CTLA-4 and anti-

PD1; combination therapy or sequential. With the combination it could potentially lead to more potent and perhaps more durable effect or even cure, but with higher risk of toxicity risking quality of life. With sequential treatment the potential risk of severe toxicity lowers but with the price of reduced potential of efficacy? These are questions we have to get more involved in and also to raise these questions with the patients. What is important to them and what are they prepared to offer and to risk for the potential longer time of tumor freedom?

1.3.2.5 Adjuvant treatment with Immune Checkpoint Inhibitors for advanced disease

Since 2017 immunotherapy with anti-PD1, both nivolumab and pembrolizumab are approved also in the adjuvant setting regarding stage III-IV melanoma that is surgically radical. Their approval was based on improved and prolonged RFS (Relapse free survival). Nivolumab in the adjuvant setting resulted improved RFS compared to ipilimumab with lower toxicities [190]. Pembrolizumab was shown to result in longer RFS compared to placebo and with no new toxicities compared to other pembrolizumab monotherapy trials [191].

While earlier the care of melanoma patients was mostly about active follow-up of stage II and III patients and treating stage IV patients with active disease, the care nowadays has shifted a lot to being more about offering treatment in the adjuvant setting. This is a total new way for us to deal with these patients. Earlier there were many questions about why there was no treatment given to reduce the risk of recurrence. Now there actually are several that reduce the risk of recurrence, but it is not certain they offer prolonged survival and they could cause durable toxicity. The patients are mostly positive to receiving adjuvant treatment though I suppose they feel that is more secure than just to wait and see. But we have to bear in mind that right now we do not know if our adjuvant treatments offer them prolonged survival and this we have to be humble to. Especially, as the treatments could mean toxicity resulting in affected quality of life.

1.3.3 Resistance to check-point inhibition

Despite the new drugs and the new strategies and their success in providing long-term efficacy and even cure for a small group of patients, not all patients are suitable for immune therapy as it is today. Around 35-60% of patients treated with anti-PD-1 respond to treatment according to RECIST criteria, but that also means that around 40-60% will not experience response and about 45% of patients experiencing response will eventually progress [167, 177, 192].
So who are the patients suitable for immune therapy? Why do some not respond at all and why do some respond initially, even during a long time and then progress?
In the clinical setting we have through experience learned that a patient with large tumor burden, rapid disease progression, elevated LDH, affected performance status or symptoms from CNS metastases is not ideal for immune therapy.

But what are the biological factors involved in response and lack of response?
Analysis of clinical data have helped distinguish between the main groups of patients and their pattern of response or the lack of thereof. The limited group that respond initially and

continue to respond (responders) (Although the responders could be difficult to define and sort out, as the response could be in a heterogeneous manner, that we have not been accustomed to). Despite that, patients benefiting from ICI seem to do so for a durable time, regardless of the heterogeneity of exact response categorization [193]). Those that fail to ever respond (innate resistance), and those that initially respond but eventually develop disease progression (acquired resistance) [194-196]. This way of describing responders is also one way to classify the type of resistance (innate or primary and required).

Resistance can also be classified as intrinsic or extrinsic to tumor cells. Intrinsic resistance is seen when cancer cells alter processes that are related to immune recognition, cell signaling, gene expression, and DNA damage response. Extrinsic resistance occur external to tumor cells throughout the T-cell activation process [197].

The exact mechanisms behind resistance to ICI and the different types of resistance are not fully understood, although the field is continuously progressing. To a large extent this depends on the fact that the exact mechanisms behind response and long term benefit from ICI are not either fully understood. Therefore it is useful to keep the known mechanisms of immune response to tumor in mind in order to understand potential mechanisms of resistance as many of the important steps of activation of immune response to tumor can be inhibited, blocked, bypassed, upregulated or hi-jacked by the tumor, contributing to resistance.

There are many theories related to the exact mechanisms of response to ICI. After having tried to get at grasp on this large, continuous expanding field I have understood that it is generally accepted that in order to achieve a successful antitumor response to ICI, a reactivation and clonal expansion of antigen experienced T cells in the TME is necessary [193, 196, 198]. In other words; optimal antigen presentation, proper recognition of these by TCR, signal one in T cell activation, signal two in T cell activation, expansion of tumor specific of CD8+ T cells that differentiate into effector T cells that migrate to TME and kill tumor cells [193, 198, 199]. For long-term immunologic memory, a part of the effector T cells have to develop into memory T cells, presumably contributing to durable responses of the disease [60, 193, 200, 201].

As this thesis is based on clinical experience and clinical studies, some mechanisms of resistance will be briefly and superficially reflected upon related to the clinical setting concerning primary/innate and acquired and in relation to melanoma resistance to ICI.

1.3.3.1 Primary/innate resistance

The patients not responding to ICI already upfront could have a disease with clinical features mentioned above and some of the molecular mechanisms will here be mentioned and they can appear at any level of the immune response to tumor discussed above or be of intrinsic or extrinsic kind.

Some tumors lack sufficient antigen presentation by the immune system [202] or do not present antigens that can be recognized as foreign [195]. The procedure to sort out normal

cells from tumor cells depends on T cells being able to recognize certain tumor associated antigens (TAA). Tumor immune evasion by TAA negative cells has been reported in melanoma patients relapsing after vaccine therapy [203].
Mutational load is a feature correlated with anti-tumor immune response and response to ICI, presumably by enhanced neoantigen formation [199, 204]. Thus tumor types harboring high levels of mutations (e.g., melanoma, lung, and bladder) are among those with highest response rates to ICI [205]. In advanced melanoma, an increased mutational burden is also associated with elevated PD-L1 expression [206]. This has been associated with response to ICI in several studies [207, 208].
To be able to properly activate T cells DCs go through maturation, where they for instance increase their expression of co-stimulatory molecules [209]. In melanoma it has been shown that the density of DCs correlates with activated T cells [210].

When the T cells have become activated they travel to tumor sites and hopefully infiltrate tumors to eradicate them. There are a number of ways tumors can prevent this from happening and thereby contributing to resistance. They can for instance downregulate chemokines necessary for attraction of T cells to the tumor, upregulate endothelin B receptor, affect the T cell homing, adhesion and migration. They are also able to overexpress VEGF (Vascular endothelial growth factor) that negatively affects T cell adhesion to endothelium, resulting in decreased infiltration of T cells into tumors [211], which has been associated with increased growth and progression in melanoma. It has also been shown that tumor biopsies from non-responders to ICI had increased expression of VEGF compared to responders [212].
When T cells have become properly activated and infiltrate the tumor they can also be stopped by immunosuppressive cells within the tumor microenvironment. These cells use several mechanisms to hinder the T cells. Upregulation of PD-L1 which leads to decreased function of cytotoxic T cells thereby helping tumors to escape. Induction of IDO, that via its degradation of tryptophane plays a role in the negative regulation and suppression of T cell function. T cells go into arrest when tryptophane is depleted [213]. An association has also been shown between IDO and recruitment of and increased infiltration of MDSCs as well as CD4+FOXP3+ Tregs, which both are known to suppress T cells [214]. Clinical trials have also shown that increased presence of MDSCs in TME was correlated to worse outcome in patients treated with ICI [215]. Upregulation of T regulatory cells leads to further suppression of T cells and as mentioned earlier Tregs suppress T cells through a number of ways, for instance directly through cell-cell contact or through inhibitory cytokines. Infiltration of Tregs has been observed in many cancer types, also among TILs in metastatic melanoma lesions, suggesting their immunosuppressive activity [216]. A correlation between decreased Tregs and increased tumor control and improved survival outcome in patients treated with ipilimumab was shown already several years ago [217].
Mutations in the molecules JAK1/2 (described below more in detail) have also been linked to primary resistance to ICI in melanoma [218].

1.3.3.2 Acquired resistance

Clinically, acquired resistance can occur in many ways. It can develop after only a short period of initial response or gradually via progression of initially only a few lesions to documented progression. It can also develop after an even longer period when a previous responder relapses, causing restart of ICI and not responding to it again.

Some molecular features involved in this and in relation to melanoma treatment with ICI will be reflected upon in the following. Many of the mechanisms underlying primary resistance are also thought to be involved in the development of acquired resistance. They can occur in analogy with what was discussed above, at different levels of the activation of immune response to tumors.

At the level of antigen presentation and activation of T cells a number of events can occur that could contribute to acquired resistance, for instance mechanisms leading to loss of expression of neo-antigens or loss in beta-2-microglobulin expression causing failure in MHC function, leading to a decrease in recognition by T cells [219]. One study of patients with melanoma found truncating mutations in ß2- microglobulin, leading to loss of MHC-I expression and acquired resistance to ICI [220].

At the level of individual cells in the TME other mechanisms contributing to resistance can happen. IFN-γ signaling, through the JAK/STAT family receptors, upregulates expression of MHC class I, contributing to enhanced antigen presentation. Mutations in JAK 1/2 have been linked to acquired resistance to ICI treatment in melanoma, as these mutations in tumor cells lead to decreased sensitivity to IFN-γ, thereby preventing IFN-γ induced cell growth arrest [220]. IFN-γ also functions within a negative-feedback loop to increase expression of PD-L1 [221] and upregulation of PD-L1 on tumor cells limits the function of T cells.

Overexpression of other immune check-points than CTLA-4 and PD-1 has been linked to resistance to ICI. In several studies a correlation between upregulation of for instance TIM-3, LAG-3 and acquired resistance to ICI has been observed (not only in melanoma) [222-224]. Other immune check-points continue to be discovered, for instance including B and T lymphocyte attenuator (BTLA), T-cell immune-receptor tyrosine-based inhibition motif domain (TIGIT), and V-domain immunoglobulin-containing suppressor of T-cell activation (VISTA). Co-expression of several immune check-points has been associated with an exhausted state of T cells and thereby perhaps a poor response to therapy. These check-points need to be further explored.

1.3.4 Cold and hot tumors

A way of describing and understanding the behavior of tumors and thereby how to treat them and make them more prone to respond to therapy, especially immunotherapy, is categorizing them as cold and hot tumors.
A “cold” tumor is characterized by a lack of immune cell infiltration in the tumor-tissue and it is surrounded by a microenvironment rich in immunosuppressive cells such as T regulatory

cells and MDSCs and poor in NK cells, CD8+ T cells and functional APC [225].
A “hot” tumor or an inflamed tumor has a high infiltration of immunocompetent cells such as CD8+ T cells and NK cells and has high numbers of functional APCs [225].
There are groups of tumors that are generally considered to be “hot” tumors and they include melanoma, kidney cancer, bladder cancer, head and neck cancers, liver cancer and non-small lung cancer. In contrast groups of tumors considered to be “cold” tumors are ovarian cancer, prostate cancer, pancreatic cancer and glioblastomas.
Within a tumor group generally known to be “hot”, there is of course heterogeneity and not all tumors within that group are “hot”.
Tumor groups considered as “hot” are more prone to respond to immunotherapy, especially anti-PD-1 therapy.

1.3.5 New treatment combinations and treatment sequence

The resistance to checkpoint inhibitors, either primary or acquired, has led to the development of combinations of checkpoint inhibitors and other treatment strategies in order to overcome the resistance. In addition to that, these combinations have the underlying intent to convert a cold tumor into a hot one, in order to make the disease more prone to respond to immune therapy.
There are almost an uncountable amount of trials going on with different combinations of checkpoint blockade and other treatment strategies. Most of them have the basis and rationale in the resistance mechanisms mentioned above, and address how new combinations can help overcome both primary and secondary resistance. Some of them have sought their way to the clinic and some of them have become a part of the daily clinical work nowadays also meaning treating patients in clinical trials with new combinations. From being almost naïve to trials as there where not so many including melanoma patients back in the “old” days (like 15 years ago.), melanoma oncologists have become wiser at including patients in new trials with agents one hardly can pronounce or even less fully understand the theory behind. Luckily there is often information for the patients in the informed consent that one could lean on.
There are several modalities trying to overcome resistance and by that trying to create better response rates and better duration of response [197]. Combination of different ICI, ICI and chemotherapy, ICI and different types of radiotherapy, ICI and targeted therapies (with one combination recently (july 2020) approved by FDA; atezolizumab in combination with cobimetinib and vemurafenib for patients with unresectable or metastasized *BRAF* V600 mutation positive melanoma), ICI and macrophage/(TAM) inhibitors, ICI and cytokine/chemokine inhibitors, ICI and epigenetic modulators, ICI and immune-stimulatory agents, ICI and oncolytic viruses, ICI and cancer vaccines or ACT which together will be dealt with further.
In this bushy landscape of trials and potential actions and efficacy it is easy to get lost, both for us clinicians and for the patients. It is therefore of greater and greater importance to keep the real effect in mind and to try not to get stuck in all the branches of new potential revolutionary treatments.

1.3.6 Biomarkers

The necessity to find biomarkers, especially in relation to ICI, has by others been described in a three-fold way, which I agree to and find wise: they may allow for a more personalized treatment, they may lead to minimizing toxicity without positive treatment outcome and finally they may save costs and resources. During the past years when the use of ICI has developed, several biomarkers have been identified and studied, but so far there is no consensus and no established biomarkers to rely fully on when deciding regarding therapy and the sequence thereof.

A biomarker is a cellular, biochemical, and/or molecular (genetic and epigenetic) characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [226]. They can be *prognostic* which offer insight to overall outcome of a patient, *predictive* that could offer insight to the probability of a response of a patient to a particular treatment or *pharmacodynamic* whose level changes in response to an exposure to a medical product or an environmental agent. Parameters used as biomarkers can be derived from blood, tumor tissue or be related to clinical or host factors or toxicity and in the case of melanoma related to irAEs.

1.3.6.1 Prognostic biomarkers

In primary melanoma, the most known and used prognostic biomarker is the thickness of the primary lesion measured in mm (Breslow) which is a part of the staging system AJCC and TNM.

The earliest and most used and well established clinical prognostic biomarker for spread disease in melanoma is LDH. Its role is to catalyze pyruvate to lactate and by that indicating cancer metabolic activity and increased glucose uptake by tumor cells. It is by that an unspecific marker indicating high metabolism and tumor burden in a variety of malignancies, including melanoma [227, 228]. It was shown by Balch et al in the beginning of the 2000's to be an independent predictor of overall survival in over 8000 patients with advanced disease. The 1-year survival rate in that cohort for patients with normal LDH was 65%, whereas only 32% for patients with elevated LDH [33]. LDH is used in the clinical setting before deciding if and what systemic therapy to choose and thereafter to follow possible treatment response or disease development.
LDH has also been shown to be a negative prognostic marker, regardless of treatment given, including the new modern treatments [148, 229-232]. Regarding ICI, LDH is in several large trials shown to be an indicator of overall outcome. For instance in the three-armed trial with ipilimumab/nivolumab vs nivolumab vs ipilimumab outcomes varied markedly by baseline serum LDH value [178].

Burden of metastatic disease has also been used when considering severity of disease and likelihood to benefit from treatment. For instance, in the trial mentioned above with ipilimumab/nivolumab versus nivolumab versus ipilimumab a subgroup analysis was

performed regarding burden of disease looking at both the summed measure of lesions at baseline and number of metastatic sites. It was shown that both the summed measure of lesions at baseline and number of metastatic sites correlated with outcome [178].

1.3.6.2 Predictive biomarkers

When it comes to predictive biomarkers regarding the new modern treatments, the research field is, as with new treatment combinations, almost flooding over. There have been some indications of biomarkers that could be indicting treatment response, but none of them really yet seeking its way into the clinical setting acting as useful markers of response to ICI, optimal regimen selection or development of irAEs.
The most famous, well-characterized and clinically used predictive biomarker in patients with metastatic melanoma is the presence of a *BRAF*V600 mutation, which is highly predictive for response to BRAF ± MEK inhibition.

When considering predictive biomarkers in regards to ICI they are often categorized as: *tissue/tumor, tumor microenvironment, blood* or *clinical*.

Tumor intrinsic parameters associated with response to ICI

Tumor size at baseline, at start of treatment with ICI, has been linked to response rate and OS, where it is more favorable to have lesser baseline tumor size [233, 234].
A higher TMB has been linked to better response to ICI [235] and it is hypothesized that it is due to increasing numbers of mutations correlating with increased neoantigen generation, which then increases the likelihood of generating immunogenic peptides that can be attacked by T cells.
Mismatch repair (MMR) is a process by which cells identify and correct mismatched DNA bases. Tumors deficient in this process (dMMR) consequently have an increased mutational burden which could be identified by the presence of microsatellite instability (MSI). MSI-high (MSI-h) has been linked to higher anti-tumor response upon treatment with ICI [236, 237]. Although it is uncertain whether it is MSI-h itself that could be linked to response, or if it is the consequence of MSI-h, high mutational burden or neoantigen formation that is the actual link.

Tumor microenvironment parameters associated with response to ICI

PD-L1 is a ligand of PD-1 and serves an inhibitory signal in PD-1 expressing cells. By that, the expression of PD-L1 (assessed by IHC staining) in tumor environment would have been an ideal biomarker and has been speculated to correlate with response in ICI treated patients. But the use of different PD-L1 IHC antibodies with non-homogenous cut-off values among the studies have led to contrasting results, making it not totally reliable as a biomarker so far [169, 177]. Although it continues to be explored as it theoretically would have been a good biomarker and as it still might offer important information considering the circumstances in which it has been used, as mentioned above.
Higher numbers of tumor infiltrating lymphocytes (TILs) have generally been a favorable

prognostic factor in many types of cancers, such as melanoma and colorectal cancer [238, 239]. The presence of CD8+ TILs at the invasive margin have been demonstrated to correlate with better tumor response and an increase in CD8+ TILs from baseline to post-treatment biopsy, has also been demonstrated to associate with tumor regression in melanoma patients treated with pembrolizumab [240].
Loss of IFN-γ pathway genes has been associated with innate resistance to anti-CTLA-4 therapy and worse overall survival [241]. Melanoma patients with higher expression of IFN-γ and IFN-γ inducible genes before start of treatment were seen to be more likely to respond to anti-PD-L1 therapy [242]. Similarly, other studies have looked at the consequences of aberrations in downstream IFN-γ signaling, such as JAK-STAT.
MHC class I and II protein complexes are responsible for tumor antigen presentation leading to the recognition by T cells (MHC-I: CD8+ and MHC-II: CD4+) and by that they are an important step of antitumor immune response. High MHC-II expression has been positively correlated with improved response rate as well as prolonged overall survival in patients treated with anti-PD-1 [243]. Low MHC-I expression on the other hand has been correlated with increased likelihood of progressive disease in ipilimumab treated patients [244].
Increased expression of ICOS, a co-stimulating molecule expressed by activated conventional and regulatory T cells, on CD4+ T cells that was sustained for more than 12 weeks after start of ipilimumab treatment has been reported to correlate with improved survival in patients treated with that particular ICI [245]. An increase in CD4+ ICOS+ T cells already after the first cycle with ipilimumab (remaining for the whole treatment course) has also been reported by our group, although we could not find any relation to survival [246].
Finally, tumor suppressive factors and cells in the stroma as IDO, MDSC's and Tregs have also (as mentioned above in relation to resistance) been linked to outcome in patients treated with ICI [247].

Blood parameters associated with response to ICI

As mentioned above LDH is a well-established prognostic biomarker. It has also been shown to be a predictor of response. One trial that showed an association of elevated baseline LDH level with worse OS in anti-PD-1 treated patients also showed that change in LDH level during treatment was significantly associated with response. In the patients with an elevated baseline LDH level, those with a partial response had a mean reduction of 27, 3% in their serum LDH level compared to those with progressive disease who had a mean increase of 39% in serum LDH level [248].
Relative lymphocyte count (RLC) at baseline has been associated with less response in ipilimumab treated patients. Patients with RLC <10, 5% had worse survival than those with RLC >10, 5% [249].
High eosinophil count at baseline have also been associated with more favorable outcome in ipilimumab treated patients and increase in absolute eosinophil count towards the end of treatment was shown to correlate with better OS [250].
MDSCs have also been associated with treatment response to ipilimumab. Low frequency of MDSCs at baseline have been associated with the higher probability of long term survival

compared to high levels in ipilimumab treated patients [249]. This correlation has also been studied by our group, although we found that the frequency of MDSCs after the first cycle of ipilimumab correlated with outcome and clinical benefit, not the baseline frequency.
Neutrophil to Lymphocyte Ratio (NLR) is used as a marker of subclinical inflammation and is also associated with worse outcomes in several malignancies. Baseline NLR and changes in NLR during treatment is associated with overall survival, progression-free survival, and clinical response in patients treated with immunotherapy. A high NLR, no matter measured prior to or during treatment with ipilumumab, was associated with worse OS, PFS, and clinical response and an increasing NLR from baseline during treatment was correlated with worse OS and PFS in ipilimumab-treated patients [251].
Increases in absolute lymphocyte count and in percentages of CD4+ and CD8+ T cells during treatment have been associated with improved survival in ipilimumab treated patients [250].

Clinical or host parameters associated with response to ICI

Some studies have demonstrated that there are gender differences in tumor immunity, which are associated with gender different anti-tumor immune responses in general [252]. Even though not many have been able to show any correlation between gender and response to ICI, meta-analyses have shown relation between gender (in the favor of men) and PFS and OS in patients treated with ICI [253]. It has been shown that males were associated with better ORR to anti-PD1 treatment [254]. In addition to this, an association between obesity and better PFS and OS at treatment with ICI (and targeted therapy), especially in men, has been shown [255].
Response to pembrolizumab in melanoma patients over age 60 has been demonstrated to be significantly higher than those below 60 years and the likelihood of response increased with age [256]. It has also been demonstrated that that ages above 65 correlated with better ORR in patients treated with anti-PD-1 [254]. But there are opposing results reported [257] why no consensus regarding age and the prediction of response exists.
Uveal and mucosal melanoma subtypes are known have a lower mutation burden (due to the fact they are not exposed to excessive UV radiation that leads to increased DNA damage and mutations) than skin melanoma and are consequently associated with low response rates to ICI. However some favorable responses have been described for mucosal melanoma to anti-PD1 [258, 259].
Visceral metastases, especially liver metastases have been associated with less response to ICI [260].
Earlier treatment might also influence the response to and outcome of ICI treatment. Several studies have demonstrated lower response rates to anti-PD-1 in previously treated patients [172]. This fact could be theoretically biologically contradictory as earlier therapies such as chemotherapy or radiotherapy could contribute to increased mutational load, increased antigen release and thereby ultimately enhanced T cell activity.
A higher gastrointestinal microbiodiversity has been associated with positive response to ICI [261]. It is shown that patients who consume greater dietary fiber and thereby have increased diversity of gut flora have better responses to ICI [262]. Patients having consumed

antibiotics, regardless of type, prior to ICI therapy have been shown to have worse outcome, but antibiotics during ICI treatment have not been shown to worsen outcome [262]. Presence of an irAE indicates that ICI has caused its intended phamacodynamic effect: immune activation. In melanoma patients treated with nivolumab those who experienced any irAE had a significantly longer OS than those who did not, with an additional benefit in OS for those with 3 or more irAEs compared with patients with none or 1 irAE [263]. Especially cutaneous irAEs, such as rash and vitiligo, have been associated with improved OS [263].

All in all the effort of trying to find biomarkers seem to be almost untiring and hopefully it will eventually result in something substantial to use in the clinical setting, as it is of great importance to reduce the use of treatment not offering help, potentially causing side effects and potential harm. Utopic the ideal biomarker for response is one that is easy to obtain, easy to analyze, easy to interpret, does not cost much and is convenient to use in the clinical setting. This means that parameters in blood that could be analyzed and used as potential biomarkers are easy and favorable to use in the clinical setting, as that does not mean much harm to patients, it could be used in clinical routine when the patients leave blood for analyzing other factors of clinical importance.

1.3.7 Additional promising but not approved treatments

Even though there is so much more to offer patients with advanced melanoma and by that also much more hope to be able to give, there is still limited hope to offer to the group of patients that do not respond already upfront or progress after initial response.

One method of treatment suitable for a limited number of these patients is adoptive cell therapy with TILs with or without DC vaccination. Adoptive cell therapy with autologous TILs can have the ability to mediate tumor regressions despite progression on ICI [264-266]. Adoptive cell therapy with TIL to lymphodepleted patients, together with IL-2, to support the persistence of the TILs, has resulted in a high rate of clinical responses, with response-rates >50% and along with that also durable responses [117]. Until recently this method was, when selecting the right type of patients, the most effective therapy of metastasized melanoma, before the combination of anti-CTLA-4 and anti-PD-1 took over this position.
Adoptive cell therapy with TIL was developed more than 30 years ago by Steven Rosenberg and colleagues at the Surgery Branch of the NCI.
Despite the high and durable responses, this treatment method has not made its way to become an approved method in clinical routine. The reason for this could be several-fold. The production of TILs is a demanding, complex process and the treatment, especially with its IL-2 support is associated with, sometimes, severe toxicity (high fever, chills, hypotension, capillary leak syndrome, electrolyte derangements). As the treatment is not approved, it is mostly used as last-line treatment. Patients with advanced melanoma progressing on approved therapy are a vulnerable group of patients as the disease in many cases could progress very rapidly and their ability to tolerate toxicity could be limited. Therefore, with the long and complex production of TILs and the sometimes harsh toxicity, far from all patients progressing on ICI are suitable for this treatment. But when selecting the appropriate patients it is, despite its toxicity profile, a very good treatment that could offer prolonged overall survival and symptom-relief beyond check-point blockade.

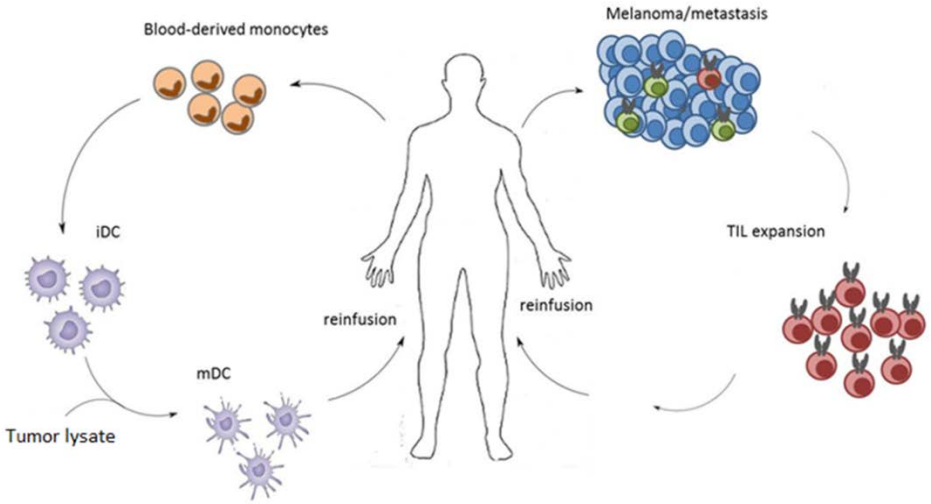

Figure 12. ACT with DC vaccination

(L. Rehn, 2017)

1.4 FINAL REFLECTIONS FROM A CLINICIAN

With all new knowledge and new treatments in mind, could my relative have had a longer period of symptom relief or even becoming free of disease, had the disease recurred later or today. It seems to me that the course of recurrence was very rapid and that his performance status along with that also deteriorated very rapidly. One could imagine that the LDH level was elevated by the time of stage IV diagnosis, so from a clinical perspective it seems like there would not have been any indication for single ICI with anti-PD1, and combination ICI would have caused a much too high risk of toxicity that he would not have been able to tolerate. If the tumor would had had a *BRAF* mutation, it would have been indicated to treat him with BRAF/MEK inhibitors, since I believe that he could have handled the toxicity and that could have given him some more time and some symptom relief. But thinking that he would have become a long lasting responder with that rapid disease progression is unlikely. Then at progression, if treated with and responding to targeted therapy, it is likely that the disease would have taken the stormy rapid course again, which often is the case, not allowing for second line with immune therapy. That fact also would not have made him suitable for ACT with TIL with or without dendritic cell vaccination since the production time would have had exceeded his survival. If there was no *BRAF* mutation, then he would have been left with exactly what he was left with, best supportive care or maybe temozolomide, which perhaps he would have tolerated, but not likely bringing him benefit. That we have to bear in mind, that even though there has been a revolution going on right in front of our clinical eyes, best supportive care, is still sometimes what we are left with. That might not even be the worst alternative considering the sometimes harsh toxicity and the risk of spending considerable valuable time in hospital. Still we are very focused on winning time and offering some type of treatment and I believe that it is of importance that we communicate with the patients so that they are properly informed what and if they might be gaining from the treatment, because perhaps all they have heard is that the “Nobel prize treatment” is like a magical solution. Perhaps the patients actually do not value time and any type of treatment more than being autonomous, but are driven by relatives or societal factors focusing on time and longevity. It is easy for us only to focus on new strategies, but with these new treatments our responsibility gets wider, we also have to inform the patients and their relatives when and if there is something to favor from the treatment. I believe that is important to bear in mind that despite the revolution sometimes we are left with what we had before the revolution and respect that as a part of our profession as well.

2 AIMS OF THE THESIS

The overall aim of the thesis is to identify mechanisms of action in the immune system that could correlate with clinical response and toxicity to treatment with ICI using anti-CTLA-4 and anti PD-1 antibodies. In addition, another aim was to conduct a phase I trial with adoptive T cell therapy with or without dendritic cell vaccination aiming for approval to offer this type of treatment to more patients.

Specific aims

2.1 PAPER I

- To study the immune system of patients with MM treated with anti-CTLA4, ipilimumab
- To investigate potential mechanisms of action that could correlate with treatment outcome and toxicity

2.2 PAPER II

- To study the immune system of patients with MM treated with anti-PD1, nivolumab or pembrolizumab
- To investigate potential mechanisms of action that could correlate with treatment outcome and toxicity.

2.3 PAPER III

- To perform a phase I trial to evaluate the safety, feasibility and immunologic response of adoptive T cell transfer with or without dendritic cell vaccination in patients with MM progressing on approved standard treatments.
- To measure how long the infused T-cells can persist in the blood.

3 PATIENTS AND METHODS

3.1 PATIENTS

3.1.1 Paper I

Forty-three patients with metastasized stage IV melanoma according to AJCC gave written informed consent to participate in the trial while treated with ipilimumab at Radiumhemmet, Karolinska University Hospital during the period of July 2012 until May 2015. The first 6 patients were included in a multicenter clinical trial, CA184-169 receiving ipilimumab at 3 or 10 mg/kg every three weeks for up to four cycles and the others were treated according to clinical routine, receiving ipilimumab at 3 mg/kg every three weeks for up to four cycles.

Peripheral blood samples for analytical purpose were collected at three time points; before the first cycle, before the second cycle and before the fourth cycle.

3.1.2 Paper II

Between February 2015 and July 2018 36 patients with metastasized stage IV melanoma according to AJCC gave written informed consent to participate in the trial while treated with anti-PD-1, either nivolumab every other week or every four weeks, or pembrolizumab every three weeks at the Department of Oncology Karolinska University Hospital.

Peripheral blood samples for analytical purpose were obtained at three time points; before the start of the first cycle, before the second cycle and before the fourth cycle.

3.1.3 Paper III

The phase I, MAT-02 trial was conducted between October 2013 and May 2018. Patients eligible for the trial were 18-74 years old, had progressive and inoperable stage III or stage IV (according to AJCC) malignant melanoma with measurable disease (according to Recist 1.1), were ambulatory in performance status 0-2 (according to ECOG) with a life expectancy of at least 3 months and had a tumor lesion available for surgery or for core biopsy. Main exclusion criteria were active CNS metastases, severe comorbidity or active autoimmune disease.

Blood samples for analytical purpose with immune monitoring were collected at several time points during the trial (see figure of description of the trial).

3.2 METHODS

3.2.1 Paper I

According to clinical routine, blood samples including complete blood count, electrolytes, creatinine, liver status and thyroid status, as well as the performance status of the patient and evaluation of any AEs were assessed before each cycle of ipilimumab was administered. The patients underwent radiological evaluation with computed tomography approximately one month after the last cycle of treatment. The response of the patients included in the trial CA184-169 was assessed according to WHO criteria and for the other patients in general according to immune related response criteria, irRC, but not in a formalized manner. AEs were graded 0-5, according to National Cancer Institute Common Terminology Criteria for AEs version 4.0 (CTCAE 4.0).

Collected blood was analyzed for counts of leukocytes, neutrophils, lymphocytes and eosinophils as well as for peripheral blood mononuclear cells. PBMCs were obtained from the blood samples by ficoll density gradient centrifugation within 1 hour of sample collection. Purified PBMCs were used immediately for flow cytometry staining and analysis or cryopreserved in fetal calf serum with 10 % DMSO. Fresh PBMCs were after proper titration stained according to the manufacturer's recommendations. Dead cells were excluded using a specific kit (see the original article). Cells were then analyzed using an LSRII flow cytometer and FlowJo software, using a non-stained control for each sample and fluorescence minus one control for critical stainings. A specific gating strategy for each of the populations analyzed was used. Quality control of the flow cytometer's performance and CV values were performed daily.

3.2.2 Paper II

According to local clinical routine, patients provided blood samples including complete blood count, electrolytes, creatinine, liver status, thyroid status before every cycle of anti PD-1 antibody and those, along with the clinical general condition of the patients and any AEs were assessed before each new cycle was administered. Patients underwent the first radiological evaluation approximately three months after the start of treatment. Thereafter approximately every three months depending on the response and the clinical condition of the patient. The radiological evaluation was not made strictly according to irRC. The evaluation of response was made by weighing together clinical outcome and radiological evaluation.

From peripheral blood counts of leukocytes, neutrophils, lymphocytes and eosinophils were measured. PBMCs were purified from the blood samples by ficoll density gradient centrifugation within 1 hour of sample collection. Regarding the staining and flow cytometry, see above.

3.2.3 Paper III

The MAT-02 trial was divided in two cohorts: cohort A where the patients were treated with T cell transfer alone and cohort B where dendritic cell vaccinations were added. Both cohorts were treated with lymphodepleting chemotherapy consisting of cyclophosphamide and fludarabine before T cell transfusion and with intravenous IL-2 after T cell transfusion for the persistence of the infused T-cells**.** Before start of the lymphodepleting regimen the patients underwent leukaferesis twice, the first time to generate material for the production of the vaccine and the second time to create a rescue bank of stems cells in case of extended leukopenia. Toxicity was assessed according to CTCAE 4.0, clinical efficacy was assessed with [18F]-FDG PET/CT and evaluated according to RECIST 1.1.

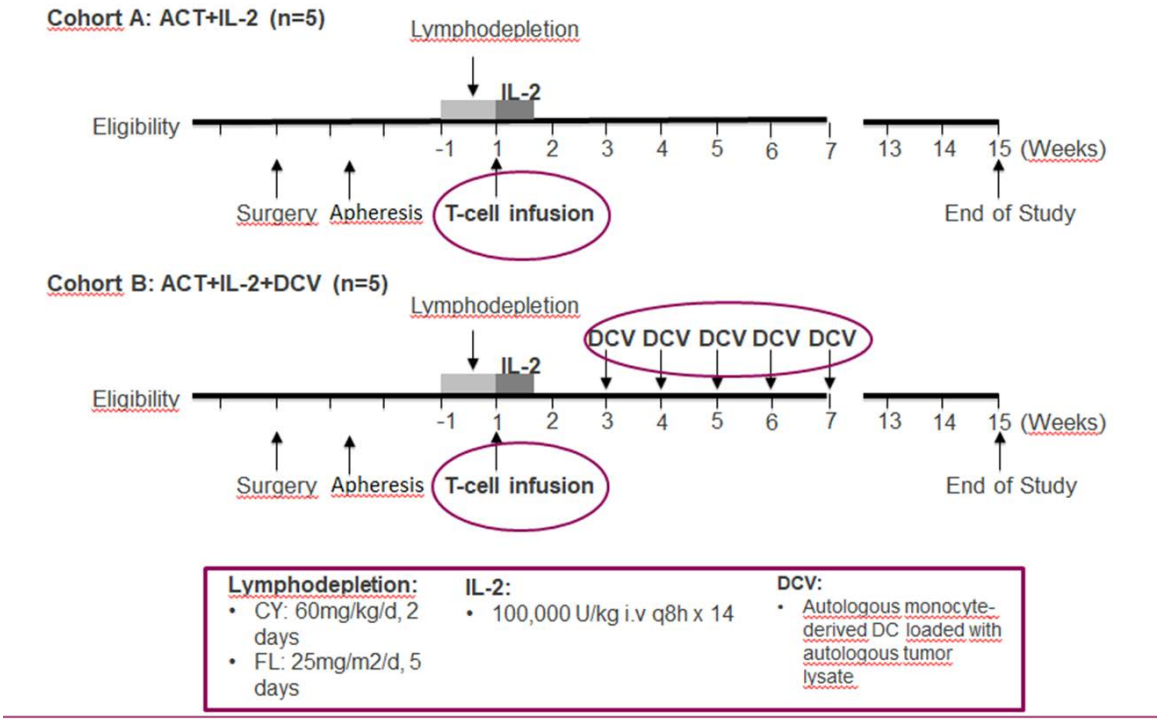

Figure 13. Overview of the MAT02 clinical trial with the cohorts A and B, timeline and procedures related to the treatment.

Autologous young TILs were expanded from one or several excised melanoma tumors, or when no excisable tumor available, retrieved from a core biopsy. Briefly, tumor material was minced into 1-3 mm^3 fragments that were put into cultures where T cell outgrowth was stimulated by addition of high dose IL-2. Once enough T cells could be collected (after 2-4 weeks), a rapid expansion protocol (REP) was performed to massively increase T cell numbers. This was done by culturing TILs with an excess of irradiated autologous PBMCs as feeder cells for 6-7 days and thereafter in WAVE bioreactors (GE Healthcare) for another 7-8 days. Harvested TIL were re-suspended in NaCl and immediately transferred to patients as an intravenous infusion.

For the production of DC vaccine, tumor lysate was generated by freeze-thawing of a tumor fragment from the same tumor that was used for TIL generation. DCs were generated from monocytes enriched from one of the leukapheresis of the patient. Monocytes were cultured for a couple of days and the immature DCs were thereafter loaded with tumor lysate and matured. A portion of the matured DCs were harvested and incubated with NY-ESO-1 and thereafter mixed together with the rest of the matured DCs. All mature DCs were frozen and on the day of the vaccination one vial was thawed, washed and re-suspended in NaCl before giving as intradermal injection.

Blood samples were collected before and at several time-points after TIL infusion. PMBSs and TILs were analyzed for T cell maturation and exhaustion by flow cytometry. TIL samples were also analyzed for T cell-specificity, using MHC-multimers for known shared melanoma TAAs.

Deep-sequencing of the TCRB (T cell receptor beta chain) gene complementarity determining region 3 (CDR3) was performed on the final TIL product as well as on PBMC samples collected before and after therapy to identify high frequency T cell clones. Frequencies of clones were analyzed and exported (Survey level ImmunoSeq; Adaptive Biotechnologies).

4 RESULTS

4.1 PAPER I

Most patients were in good general condition at treatment initiation with ipilimumab. The median overall survival was 39 weeks. Just above 60% of the patients could receive all four cycles and the most common reason for not receiving all four cycles was AEs. More than half of the patient cohort experienced AEs of any grade and almost one third experienced AEs of grade 3 or higher. The AEs could in general be managed with withdrawal of treatment or corticosteroids or in a few cases with TNF-alfa antagonist, not causing any long-lasting sequel for most of the patients. The objective response rate was 19% with no patient obtaining complete response. The patients were divided in two groups for analytical purpose, those with clinical benefit (response and stable disease) and those with no clinical benefit (progressive disease). The patients with clinical benefit had significantly longer median overall survival.

Absolute lymphocyte counts, eosinophils, effector T cells and their activation status were increased in the patient cohort. Additionally, suppressor cells such as T regulatory cells and polymorphonuclear myeloid derived suppressor cells (PMN-MDSCs) were diminished and these effects were observable at the end of treatment and after one cycle, respectively. Total eosinophil count correlated to onset of AEs. Monocytic MDSCs decreased during treatment in patients with clinical benefit and patients with a lower frequency of these cells after the first cycle had increased overall survival. CD8+ effector memory T cell frequencies at the end of treatment were higher in patients with clinical benefit and correlated with longer survival.

4.2 PAPER II

At the time of treatment initiation with anti-PD-1antibodies most patients were in good general condition, 39% had elevated LDH and 69% were treatment naïve. At the time of data cut-off the majority of patients had died (47%). Median overall survival was 126 weeks and median progression free survival was 38 weeks. Objective response was seen in 50% of the patients out of which 28% had complete response and 22% had partial response and 36% of the patient cohort had progressive disease. 36% of the patients discontinued treatment due to complete response or durable stable disease. Patients were divided into short PFS (<6 months) and long PFS (>6 months). 20 patients had long PFS and among these 10 with CR, 7 with PR, 1 MR, 2 SD and the median overall survival for the patients with short PFS being 45 weeks and for the patients with long PFS 170 weeks. Treatment related adverse event of any grade occurred in almost 60% of the patients and grade 3 or higher in almost 15%. The AEs were in general easily managed with corticosteroids.

No significant changes were seen in absolute counts of leukocytes, lymphocytes, eosinophils, neutrophils or monocytes. The number of neutrophils and monocytic MDSCs correlated with survival. It was observed that patients with short PFS had higher levels of neutrophils and

high number of MDSCs at baseline. It was also observed that patients with high number of MDSCs at baseline had correspondingly shorter median OS. In addition, it was observed that patients with low frequencies of CD69+ NK cells at baseline had both longer PFS and OS. PD-L1 expression in different monocytic subsets was significantly increased in patients with shorter PFS and correlated inversely with OS.

4.3 PAPER III

Of 14 patients with progressive metastatic melanoma enrolled in the MAT-02 study 10 were treated. All enrolled patients except one had received and progressed on ICI.

In cohort A, all treated patients showed a mixed response or stable disease. However, these responses were not durable, with an overall survival ranging between 4 and 17.5 months.

In cohort B, five patients were enrolled and commenced therapy as per protocol. One patient died before receiving the DC vaccine and was not evaluable for response. Of the four patients receiving the complete combined TIL and DC -treatment, all had an objective response according to RECIST 1.1. These responses consisted of two durable complete responses, one durable high-quality partial response and 1 short-term partial response. The short-term response was only considered a mixed response by irRC.

All 10 patients received the lymphodepleting chemotherapy.

The majority of patients reacted with mild to moderate toxicity, such as fever and chills following the TIL transfusion which often was successfully treated with paracetamol.
The most severe AEs in the trial occurred in response to IL-2 therapy. In cohort A, four of five treated patients received all 14 doses of IL-2, whereas in cohort B, none of five patients received all doses. The main reason for stopping IL-2 infusions was capillary leakage, a known side-effect of IL-2 therapy.
One patient died during the course of treatment. After ending the IL-2 treatment the patient suddenly desaturated and was treated at the intensive care unit. Despite all at the intensive care unit resources the patient died, most likely due to metabolic acidosis, a known but rare toxicity of IL-2 treatment.
None of the four patients in cohort B receiving the DC-vaccinations experienced any particular toxicity to these injections.

The production of TILs was possible for all patients. The median production time for TILs was 35 days. The median yield given back to the patient was 10,3E9 for cohort A and 42E9 for cohort B. The production was however less successful for 3 patients in cohort A, which in all three cases was due to slow expansion in the REP step. The viability of the T cell product at final harvest was high (mean 97%), and the cultures from most of the patients were predominantly T cells, with a strong skewing towards the CD8+ subpopulation.

Patients in cohort B received a tumor lysate-loaded DC-vaccine produced from monocytes enriched by elutriation from a leukapheresis product. The enriched product contained 93% monocytes. After DC differentiation and tumor lysate-loading, mDC showed a mature phenotype with high viability. Four patients in cohort B received five doses of intradermal DC vaccine with high viability. The effect was clinically assessed by a DTH test for the tumor lysate and the NY-ESO-1 peptide. None of the patients reacted to the tumor lysate, but one patient reacted with skin reaction to the NY-ESO-1 peptide.

An important factor for reaching any efficacy from ACT is the number of infused cells and their longevity. To study this, as mentioned above, both immune monitoring and deep sequencing of the TCRB gene were performed, with the intention to monitor the infused TILs.

The TCRB sequencing showed that the infused cells survived for a durable period and that the survival was the longest among the cells infused to patients experiencing partial response with long duration, probably due to continuous proliferation in response to the remaining tumor burden. Immune monitoring data revealed that the TILs contained both CM and EM T cells, but that they after the infusion continued to differentiate with an increase regarding the EMRA population and a decrease regarding the CM population, most probably due to that the cells came in contact with tumor cells and later also DC from the vaccination. In accordance with this, the expression of the activation and exhaustion marker PD-1 increased in most patients in T cells in blood following ACT. Increased expression of PD-1 could be a sign of exhaustion of the cells meaning a negative effect on the treatment, but despite many of the responding patients had an increase in the PD-1 expression, none of them has yet revealed any sign of progression.

5 CONCLUSIONS, DISCUSSION AND IMPLICATIONS

5.1 PAPER I

The clinical efficacy of the treatment was in concordance with what has been shown before and what is a general clinical opinion when using ipilimumab as single agent. We found that immune cells other than T cells are important during check point blockade with anti-CTLA-4 and that effects on those cells correlated with outcome. Even though ipilimumab does not have the same position in the clinical setting as it had in the beginning after the approval, currently the use of it together with or sequentially with anti-PD-1 is in practice. Our data could potentially help guiding the clinical decisions, by observing the number of MDCS cells and the number of CD8+ effector memory T cells at different time points, at baseline, after the first treatment and towards the end of ipilimumab treatment in order to guide if another drug should be added or if it is time to end treatment. If, for instance the decision to start ipiliumumab after progression on anti-PD-1 is taken, the situation could be that of a vulnerable patient in progression, not tolerating extensive toxicity and potentially not benefitting of another ICI. Then MDSCs after the first treatment could help to guide continued treatment, if they do not go down compared to baseline (before ipilimumab) then perhaps considering to halt the treatment is in place, as our data implicates that patients with low MoMDCSs already after the first cycle are the ones benefitting from ipilumab. If anti-PD-1 is considered after progression on anti-CTLA-4, the number of CD8+ effector memory T cells could be monitored to, in addition to the performance status of the patient, be of guidance in how to proceed with therapy. If the numbers of CD8+ effector memory T cells are low and the patient is in weak general condition, then perhaps it is time to start to consider switching to targeted therapy (if there is a *BRAF* mutation) instead, as our data implicated that patients with low CD 8+ effector memory T cells towards the end of ipilimumab treatment in addition to not benefitting from the treatment also had worse overall survival. If the numbers of CD8+ effector memory cells on the other hand are high but the patient is still in stable clinical condition, then one perhaps could have the courage to “wait and see”, before starting another line of therapy.

5.2 PAPER II

Also in this investigation we could observe that the clinical efficacy of the treatment with anti-PD-1 was in coherence with what has already been reported. Although more patients have benefit from the treatment with anti-PD-1 and although the number of patients experiencing limiting toxicity is lower than with the treatment with anti-CTLA-4, there is still a considerate number of patients, not experiencing efficacy. It is of great importance to find biomarkers that reliably and at low cost could allow for pretreatment selection, so that treatment could be offered only patients who will benefit. It is also of great importance to find biomarkers that are easy to use in the clinical setting. Routine hematological evaluation is one of the ideal sources of predictive and prognostic biomarker due to the fact that it incorporated

in the clinical routine, does not put the patients at any direct risk and is of low cost. In this work we could show that certain cells extracted from routine blood work and further prepared and analyzed could be interesting as potential biomarkers, although further exploration and correlation to the clinical outcome is needed. We also found in this work that other cells than T cells, such as subtypes of NK cells and subtypes of MDSC's are involved in the blockade of anti-PD-1 and that they after further exploration, perhaps could be used as potential biomarkers. These results, if they could be further explored and taken into practice means that the cooperation between the laboratory world, the bench, and the clinical world is of certain importance and that translational work and cooperation, even in in the caretaking of the patients is of great importance.

5.3 PAPER III

The MAT02 trial could be conducted in a safe way with limited and expected toxicity and it was shown that this type of treatment can be offered to selected patients with advanced melanoma having progressed on approved therapy including check-point inhibitors, regardless of earlier response to check-point inhibitor therapy. The selection of patients has to be thorough, as one patient unfortunately succumbed after IL-2 treatment. This sheds light on the importance of the selection of patients and on the time point when this treatment should be offered. At present the TILs are generated from material obtained when the patient has progressed on approved therapy. This could, in order to improve the trial and perhaps the outcome, be performed earlier. The optimal scenario would be to take tumor samples from patients with available lesions before starting check-point therapy and to save the material for possible later use for ACT therapy. This would make the production time shorter when the patients have progressed and would minimize the risk of the patients progressing during the production time. Perhaps this therapy could be offered earlier to a selected number of patients, possibly before starting check-point therapy. That would mean that the selected patients are in better performance status and hopefully enable clinicians to handle the toxicity more efficiently. If there is a good response which is followed by progression at a later time, the treatment could perhaps be offered again. If the situation is opposite, the patient could then be treated with other approved immune therapy or even targeted therapy and perhaps having a more favorable situation of response.

6 FUTURE PERSPECTIVES

When having taken part of a revolution and having the situation before and after that revolution in memory it is easy to become blinded by the progress the revolution has led to. Remembering the patients with galloping melanoma and remembering the feeling of powerlessness not being able to offer them anything could perhaps to some extent make us blinded by the success of the new groundbreaking therapies, by the fact that we actually have something to offer, no matter the result and the dark side. However, it is important to bear in mind that a revolution does not take place at all levels and that there are costs behind it.

The therapies will continue developing and we have to be careful that we do not get lost in the jungle of development and forget the patients behind the disease. We have to be cautious in order not to be blinded by the new strategies that will develop just because we remember how it used to be when there was nothing to offer. It is important to really consider what and how much the therapies really offer the patients so that we do not end up offering them treatment just because we are able to. It is important that we continue to keep our heads clear and cold so that we do not get carried away, forgetting the humans behind the overall survival numbers and not forgetting to consider if the numbers really are that good. We could be balancing on a very thin line and it is easy to fall if we get carried away not considering when to stop treating. Therefore, it is of importance to repeatedly discuss these matters, to repeatedly reconsider our decisions and to examine our decisions and why we decide in a certain way.

It is also important to consider the societal costs for these new strategies, and the resources they take up, both economically and ecologically. I believe that the burden these new therapies take up, both economically and resource-wise are something that we will be discussing in the future more and more. For how long will we be able to offer these treatments to this limited group of people? Must we not start thinking about reducing the incidence? Can we defend these treatments if we do not try to minimize the people getting the primary disease? (Maybe it is time to take action with the summer tours, caravaning the beaches again?) I believe that we have to start taking our responsibility as oncologists to a wider perspective, not just offering the patients more and more treatments or trying to fit them into more trials offering more and more combinations. Instead we have to broaden our way of working and consider primary prevention, toxicity, time spent in hospital, quality of life during treatment to greater extent and put the rates and data from the trials in a wider consideration, are they really that good considering the toxicity, the hospitalizations and the costs?

Of course, the new revolutionizing treatments already approved are a true revolution but considering what has been reflected upon above we continuously have to question and respect the treatments and what they come with and what price is paid in the shadow of the treatments.

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you lose the people closest to you that you think you could never cope without, you come to insights you never thought you should, you have to make decisions so difficult that you never thought you could make them, you come to have emotions and feelings you never thought would happen to you or that you were capable of and you feel joy, happiness and ease now and then. All this contributes to your being and molds you to who you are. But to be able to cope with many of the episodes that make up your life, you need the support from family and dear friends. I am very grateful for having so many of these that have been there with me and for me at many of the episodes making up my life. These family and friends have, together with the episodes, contributed to who I am and thank you all for that.

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