Targets for personalized treatment of conjunctival melanoma

Cao, J.

Chapter 1

Introduction and aims of the thesis

Melanoma is a type of cancer that originates from melanin-producing cells that are derived from the neural crest cells [1]. Melanoma frequently occurs in the skin, but the eye, and the mucosa of head and neck may also be involved. Ocular melanoma, the second most common type of melanoma, consists of two types: 1. those that develop in the conjunctiva, the tissue that covers the eye, and 2. those that grow intraocularly in the uvea (iris, ciliary body and choroid). This thesis focuses on the first type, the conjunctival melanoma (CM), which arises from the melanocytes within the basal layer of the conjunctival epithelium, and accounts for 5-6% of all ocular melanoma [2,3]. CM occurs mainly in the non-Hispanic white population, and is rare in young people [4]. The annual incidence is increasing and is now up to 0.8/million in Caucasians [5]. Between 1973 and 1999, the incidence in the United States increased by 295% in white males [6]. The risk factors of CM are not well understood. However, the population-based studies in Northern Europe suggest that, like cutaneous melanoma, the increasing incidence of CM is possibly associated with increased exposure to ultraviolet light radiation [5,7].

Malignant CM originates in primary acquired melanosis (PAM) (74%), de novo (19%), or in a pre-existing nevus (7%) [8]. CM may occur in any area of the conjunctiva, such as the bulbar, palpebral, caruncular, limbal and forniceal conjunctiva, and the cornea (Figure 1). CM frequently (up to 60%) recurs locally after primary treatment [9], and can be lethal because of metastasis. Intraocular extension may occur after primary surgical removal [10]. The first site of metastatic CM is often in the preauricular, submandibular or cervical lymph nodes after diagnosis of an invasive melanoma lesion, suggesting lymphatic vessels may be the main route of regional dissemination of CM [11]. However, distant metastasis may develop without first having a regional metastasis [9]. The lung, brain, liver, skin, bone, and gastrointestinal tract may be sites of distant metastases. The incidence rate

of metastatic CM varies from 12% to 25%, and the melanoma-related mortality at 10 years varies from 13% to 32% [5,9,12-14].

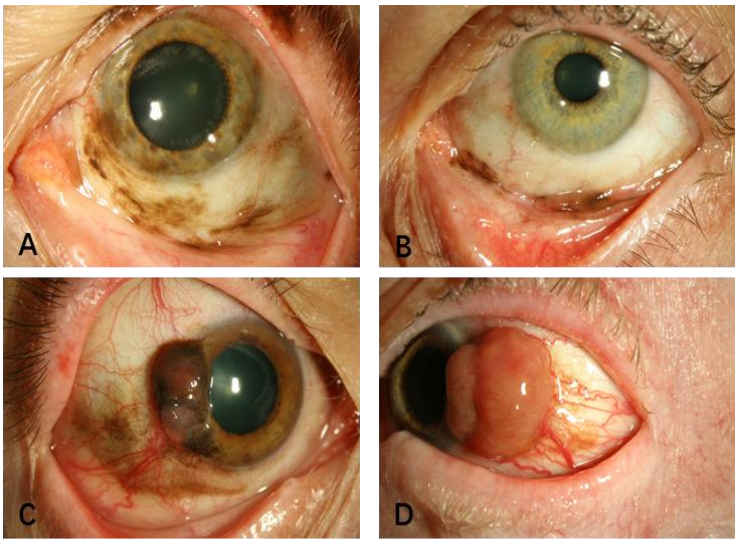

Figure. 1: **Conjunctival lesions**. **A**. Extensive primary acquired melanosis involving half the ocular surface and the corneal limbus. **B**. Pigmented CM in the fornix. Large pigmented (**C**) and non-pigmented (**D**) CM overlaying the corneal limbus.

Predisposing factors for malignancy

Conjunctival nevi, both pigmented and non-pigmented, are often found in childhood; they may be quite bulky. Although this type of lesion frequently carries a *BRAF* V600E mutation [15], it rarely progresses into melanoma [12]. On the other hand, the flat pigmented lesion known as PAM, usually detected in adults, indicates an increased risk of progression to malignant melanoma. A greater extent of PAM and/or PAM with severe atypia may potentially increase the chances of malignant transformation [16,17].

Prognostic factors

Many attempts have been made to elucidate the risk factors that predict the prognosis of CM. Firstly, the origin of melanoma may be an indicator of metastasis and death: Shields et al. reported that de novo melanoma have a higher risk of metastasis and mortality than tumors derived from PAM and nevi [8]. The presence of PAM with atypia also correlates with the development of local recurrences after biopsy or cryotherapy treatment [18]. Secondly, tumors located in the non-bulbar regions, i.e. the fornix, caruncle, or palpebra, and those with positive pathological margins are associated with local recurrence and metastasis after primary surgical treatment [8], and with a poor survival [5,9,19,20]. Thirdly, tumor thickness has prognostic value: an increased risk of local recurrence is observed in melanoma with a thickness over 0.5 mm [21], and more metastases and melanoma-related deaths are seen in thicker tumors [12,22,23]. Correspondingly, tumors thinner than 1 mm carry a low risk of mortality [9]. Lastly, other features, such as excision without adjuvant therapies, a large basal diameter, eyelid involvement and tumor lymphangiogenesis have been associated with worse clinical outcomes [5,9,11,24].

Treatment of conjunctival melanoma

Treating conjunctival lesions may start at the level of PAM, because, as we mentioned above, PAM is associated with an abnormal vascularity and melanoma transformation; Gloor and Alexandrakis therefore recommended surgical removal of widespread and thickened PAM [25]. However, the use of topical chemotherapies using mitomycin C or interferon alfa-2b without surgical excision have in recent years shown their effectiveness in decreasing the pigmentation and presence of PAM [26-28]. Regarding primary CM, extensive surgical excision used to be the conventional treatment. Whereas, growing evidence has shown a high local recurrence rate after surgery only [29,30]. Consequently, the current trend is towards surgical excision combined with adjuvant therapies.

Adjuvant therapies for CM are comprised of cryotherapy, topical chemotherapy and radiotherapy. Historically, cryotherapy was applied on the surgical margins after excision. However, Damato and Coupland found a significant higher recurrence rate and many complications after cryotherapy [31]. Therefore, cryotherapy has almost completely been replaced by topical chemotherapy as an adjuvant approach [32]. The most favorable topical drug for intraepithelial and superficial CM is mitomycin C, which has been investigated in several series [26,33,34]. Another topical drug is interferon α-2b with a treatment benefit shown in a few studies, in rather small cohorts [27,28,34]. The ideal dosage of mitomycin C and interferon α-2b are dependent on the clinical experience of doctors since there is no golden standard yet. Despite this adjuvant treatment, high recurrence rates, ranging from 33%-50%, are still being recorded after topical mitomycin C application [34-36].

Unlike topical chemotherapy, adjuvant radiotherapy may be applied for deeper malignant conjunctival lesions. Plaque brachytherapy significantly decreases the recurrence rates [30]. Similarly, proton beam radiotherapy has been applied to patients with non-bulbar primary CM. However, Wuestemeyer and colleagues demonstrated a 30% recurrence rate and 30% metastasis rate of CM upon proton beam radiotherapy [37].

Preventing patients from developing metastasis is a dilemma for treating CM. The traditional chemotherapies have limited effects, and because of the rarity, patients with advanced CM are often not included in clinical trials evaluating the effectiveness of novel therapies.

Animal models

The rarity of CM significantly limits the possibility of recruiting enough patients for randomized clinical trials to test new therapies. Therefore, it is urgent to develop animal models which can properly mimic the original disorder. However, unlike uveal melanoma, only a few models have been introduced to study CM. Successful models for CM are restricted to mice, while a model of PAM has been established

in rabbits [39]. De Waard and colleagues [38] recently developed a murine CM metastasis model, using nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) IL-2 $r\gamma^{null}$ mice in which three human primary CM cell lines were subconjunctivally injected. Although tumor growth was observed in all cases after this approach, no metastasis occurred. Subsequently, cells obtained from the subconjunctival xenografts were inoculated into the subconjunctival space of a second group of these immunodeficient mice, and this *in vivo* passaging method led to frequent lung metastases. Schlereth et al. [12] implanted two murine melanoma cell lines subconjunctivally into C57BL/6N immuno-competent mice, and observed successful primary tumor growth as well as metastases in the lymph node, lung and spleen. In the future, more attention should be paid and more effort should be taken to establish ideal models for CM.

Genetics of conjunctival melanoma

Cancer is a human genetic disorder. Investigations have shown that CM, distinct from intraocular uveal melanoma, shares many molecular similarities with cutaneous melanoma; *BRAF* and *NRAS* are both known oncogenes that contribute to the formation of cutaneous melanoma. Activating mutations in *BRAF* or *NRAS* (mutually exclusive) are frequently detected in CM as well, and have been identified in up to 47% of all CMs [40-42]. Similar to cutaneous melanoma, more than 90% of mutations in the *BRAF* gene in CM occur at the V600E position.

BRAF is a crucial component in the mitogen-activated protein kinase (MAPK) pathway, while NRAS may participate in activities of the MAPK and PI3K/AKT pathways. Tumor-driven mutations in *BRAF* and *NRAS* genes activate MAPK and PI3K/AKT pathways, thereby promoting intracellular signaling, cell proliferation and cell cycle dysregulation (Figure 2).

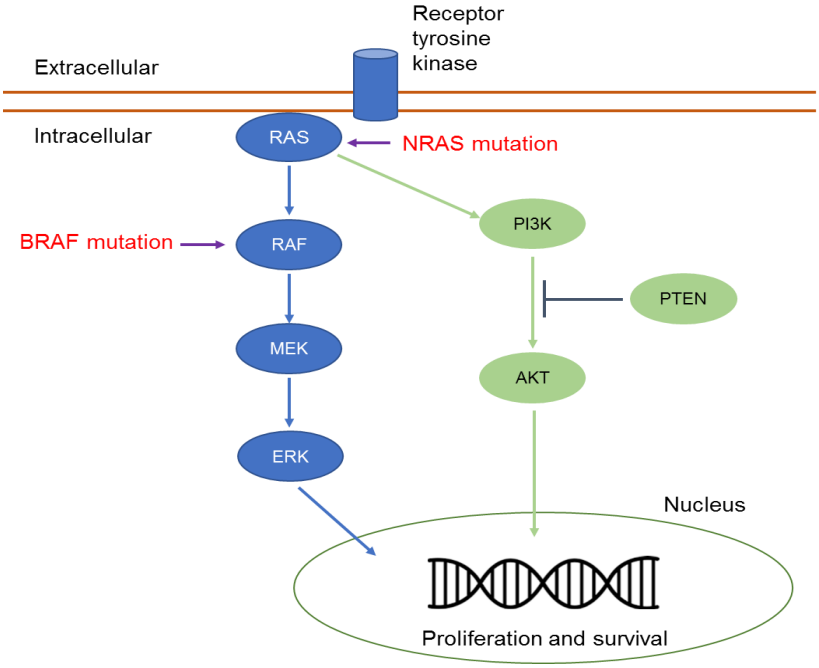

Figure 2. MAPK and PI3K/AKT signaling pathways. Oncogenic BRAF and NRAS mutations lead to phosphorylation of ERK and AKT, thereby activating the MAPK and PI3K/AKT pathways and stimulating cells to grow into a malignancy and survive.

Mutations in *GNAQ* or *GNA11*, which are oncogenes in uveal melanoma, however, are not found in CM, indicating different oncogenic triggers in these two types of ocular melanoma. Interestingly, in a large series, the *BRAF* V600E mutation was detected in conjunctival nevi and melanoma, but not in PAM with or without atypia [16]. One may speculate about the role of the *BRAF* V600E mutation in melanoma transformation, because in contrast to PAM with atypia, conjunctival nevi rarely develop into melanoma. We believe that additional mutations or oncogenic factors promote this tumor formation. Activating mutations of another hotspot oncogene of cutaneous melanoma and mucosal melanoma, KIT, are seldom identified in CM [43]. In contrast to activating mutations, abnormalities of tumor-suppressor genes like *p53*, *p16* and *PTEN* have not been well characterized in CM.

One study using a monoclonal antibody and immunohistochemistry has demonstrated a significantly lower protein expression of p16 in CM, compared to nevi and PAM with or without atypia [44], and suggested p16 expression could be used as a diagnostic marker to distinguish nevi and PAM from CM.

Many chromosomal aberrations have been discovered in CM, such as gain of 1q, 3p, 6p, 7, 17q and loss of 9p, 10q, 11, and 12q, 16q [45-47]. These biologic data are of great importance to further understand the basis of this malignancy, and to promote the development of new therapies.

Targeted therapy

The need for developing new treatments for advanced CM is urgent since the current approaches to treat metastases have limited effects. The new era of cancer personalized treatment involves comprehensive genomic or immunohistochemical analysis, thus unlike conventional chemotherapy, the new types of targeted therapies can attack tumor cells precisely with little damage to normal cells. The goal of personalized medicine is to tailor suitable therapies with the right medical decisions and medicines, at the right dose and at the right time in the right patient [48]. In cancer, personalized medicine will create more specific approaches to the individuals and their genomes. Early diagnoses and better drug development are believed to be achievable by personalized medicine. As we described above, the mutant genes in the MAPK pathway lead to abnormal activation of cell growth and survival. Therefore, personalized treatment with therapeutic agents aimed at these specific oncogenes is being applied in several types of cancer and has shown promising clinical outcomes [49]. However, only a few studies have evaluated the potential benefit of targeted therapies in CM. Pahlitzsch and Maleka et al. showed induction of good clinical responses in primary and metastatic CM patients upon Vemurafenib treatment [50,51], whereas Weber et al. reported a failed case using Vemurafenib for a metastatic CM patient with a *BRAF* V600E mutation [52]. We encourage clinical trials to recruit more CM patients using targeted compounds.

Other therapeutic approaches involve epigenetic changes: the term “epigenetics” refers to heritable changes which are not caused by alterations in the DNA sequence [53]. Aberrant patterns of epigenetics are considered a hallmark of cancer. Not only mutations and chromosomal translocations, but also aberrant chromatin-related proteins may lead to alterations in gene expression and signaling pathways, and thus result in cancer formation. The dynamic feature of epigenetic enzymes offers the opportunity to develop new drugs for cancer therapeutics. Enhancer of zeste homolog 2 (EZH2) is a core subunit of the polycomb repressive complex 2 (PRC2) family, and is of importance in regulating cellular processes. EZH2 catalyzes trimethylation of lysine 27 in histone H3 (H3K27me3) and results in the transcriptional repression of target genes [54,55]. Heterozygous somatic mutations of EZH2 are often observed in diffuse large B cell lymphomas and follicular lymphomas [56], but is not frequently found in solid tumors [57]. However, overexpression of EZH2 has been detected in many cancers, including cutaneous melanoma [58]. Targeting EZH2 with specific small molecule inhibitors has shown a high *in vitro* and *in vivo* potency against EZH2-mutant or EZH2-wildtype cancers [59]. CM may be a good target for blocking EZH2 with genetic or pharmacological approaches to inhibit cell growth *in vitro* and in *vivo*. We believe more potential molecular targets will be discovered with the evolvement of cancer research.

Immunotherapy

Immunotherapy boosts the activity of a person’s immune cells to fight against malignancies. It can be done by either stimulating the immune system or giving humanized antibodies. In the last few decades, immunotherapy has gained a revolutionary success in treating some types of cancer, especially advanced cutaneous melanoma.

Cytokine immunotherapy (with interferon and interleukin-2) has been applied for cutaneous melanoma patients, providing some good responses. However, many

complications have been noticed with this approach, making this method less favorable compared to other options [60].

The discovery of immune checkpoints is a milestone of immunological research. Immune checkpoints switch signals on or off, and cancer usually protects itself from T cell attack by blocking T cell signals. For example, when the T cell surface receptor known as programmed cell death 1 (PD-1) binds to programmed death ligand 1 (PD-L1) on the surface of a cancer cell, this will suppress the T-cell's function, impair cytokine production, and induce T cell apoptosis. To reverse this immunologically-non-responder state, antibodies against the inhibitory checkpoints have been developed, which target PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTL-A4), with promising results [61,62]. Very recently, Nivolumab (PD-1 inhibitor) showed a successful clinical response in a CM patient with breast metastasis [63]. However, very few clinical trials have recruited CM patients. It is therefore essential to study the expression of these ligands in CM.

The human leukocyte antigen (HLA) complex plays a pivotal role in immunology: cytotoxic T cells (CTL) function when HLA class I molecules are present. Cancer cells attempt to escape immune surveillance in many ways, one of which is downregulating HLA class I expression, which helps to avoid an attack from CTL. Downregulation of HLA class I expression usually is a predictive biomarker of poor prognosis in many cancer types, although opposite outcomes have been reported in colorectal cancer and uveal melanoma [64-66]. T cell receptor (TCR)-based immunotherapies have been extensively studied in the past years, especially in cutaneous melanoma. In this context, HLA class I is required to present tumor antigens to $CD8^+$ cells, letting cytotoxic $CD8^+$ cells kill tumor cells. Therefore, it is worthwhile to examine the HLA class I status in CM to help determine the therapeutic options. However, not all melanoma may express the same target antigens. A special interest focuses on the identification of T cell clones that recognize tumor-specific antigens. One study showed that a new cancer-testis antigen PRAME (Melanoma antigen preferentially expressed in tumors), is highly expressed in CM [67]. As the LUMC has extensive experience with this antigen, we

decided to test the newly designed PRAME-specific HLA-A2 restricted T cell clones on CM cell lines, and found that the HLA-A*02:01 transduced melanoma cells could be recognized by those PRAME-specific T cell clones, indicating a potential approach for treating CM (Figure 3). However, T cells only respond when the appropriate HLA antigen is expressed on the target, and we therefore studied expression of HLA class I antigens on a series of CM.

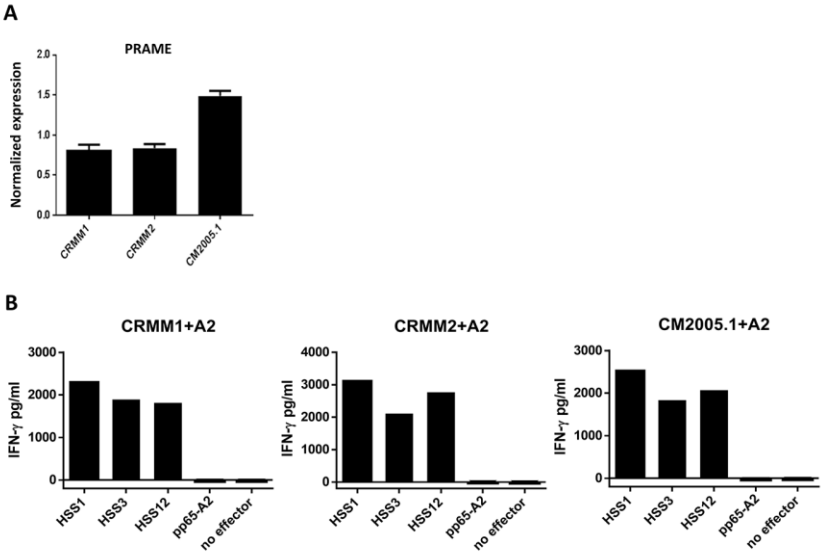

Figure 3. Three PRAME-positive CM cell lines are efficiently recognized by PRAME-specific T-cells. (**A**) The mRNA expression of PRAME in three CM cell lines (CRMM1, CRMM2 and CM2005.1) was determined using quantitative real-time polymerase chain reaction (qPCR). (**B**) The PRAME-specific T-cell clones HSS1 and HSS3 identified all CM cell lines. CM cell lines had been retrovirally transduced with HLA-A*02:01 (+ A2). USP11 (housekeeping gene)-specific T-cell clone HSS12 was used to verify that HLA-A2 was positive in CM cells. Clone pp65-A2, reacting with a peptide of pp65 in the context of HLA-A2, served as the negative control. Data are provided by DM van der Steen and MHM Heemskerk, Department of Hematology, LUMC.

There are several new directions in the immune-oncology field. For instance, dendritic cell (DC) vaccines can enhance $CD8^+$ T cell response to neoantigens (encoded by tumor-specific mutated genes) in cutaneous melanoma [68], and combining DC vaccines with immune checkpoint blocking therapy may be a future regimen [69]. Another promise lies in the combination of epigenetic therapy and

immunotherapy to enhance the immune system and thereby improve the outcome [70]. In addition, chimeric antigen receptor T-cell (CAR-T) therapy is a global focus for cancer treatment. T cells extracted from the tumor-bearing patient are genetically reengineered to express CAR that recognize a specific tumor antigen, and are subsequently reinfused into the patient. CAR-T therapy was initially designed for hematopoietic malignancies, but currently, a great effort targeting solid tumors is ongoing, although many obstacles still need to be overcome.

Aims of this thesis

Two different yet related topics are described in this thesis. The first part of the thesis identifies specific targets, illustrates the role of these targets, and explores personalized therapeutic possibilities to inhibit tumor growth by using new treatment strategies. The second part starts with the description of a new animal model for CM. A proper model can mimic the human disorder and provide opportunities for studying tumor biology, and for seeking novel effective therapies to inhibit the dissemination of malignant cells. Subsequently, the potential role of immune checkpoint molecules and the presence of HLA class I antigens are studied.

New animal models and therapeutic targets will broaden our understanding of CM, and help us to develop better treatments for primary and metastatic CM, thus prolonging patients' survival.

Scope and outline

Given the challenges described above, this thesis aims to discover targets of personalized treatment for CM. Figure 4 illustrates the different aspects of this thesis. *i*) targeting oncogenes and epigenetics, *ii*) establishment of a murine metastatic CM model and *iii*) studying the immunology and potential of using immunotherapy for CM.

In **Chapter 2**, we examine the presence of *BRAF* V600E mutation, and the

expression of phosphorylated-ERK and phosphorylated-AKT on sections of human conjunctival lesions by immunohistochemistry. We show that *BRAF* V600E mutations occur frequently in conjunctival nevi and melanoma, and ERK and AKT are expressed in these lesions as well as in PAM. Furthermore, we treated human CM cell lines with BRAF, MEK and AKT inhibitors, and reveal that although a single drug can inhibit cell growth, it is not always cytotoxic. We demonstrate that *in vitro* BRAF inhibitors (Vemurafenib and Dabrafenib) are effective on *BRAF* V600E-mutated CM cell lines, while the MEK inhibitor MEK162 and AKT inhibitor MK2206 work on all cell lines, regardless of mutation status. The combination of MEK and AKT inhibitors results in more cell cycle arrest and cell death.

Another area of targeted therapy lies in epigenetic regulators. The dynamic and versatile character of epigenetics makes seeking epigenetic targets attractive. In **Chapter 3**, we focus on the crucial epigenetic gene EZH2, and observe frequent overexpression of EZH2 in primary CM. Inhibiting EZH2 with genetic knockdown or small molecule EZH2 inhibitors impairs CM cell growth and EZH2-mediated protein expression and induces cell cycle arrest *in vitro*. Additionally, one EZH2 inhibitor reduces cell proliferation in a zebrafish model. These findings suggest that targeting EZH2 may provide an alternative treatment for CM.

To facilitate the understanding of the tumor behavior and testing potential therapeutic compounds, we created a mouse metastatic CM model (**Chapter 4**). Our model mimics the way of metastatic spread of human CM, thereby especially providing opportunities for novel treatments to prevent metastatic dissemination.

As resistance to targeted therapies may develop, it is worthwhile to look for alternative therapeutic targets. Therefore, to determine whether blocking the PD-1/PD-L1 axis can be a potential treatment, we examine the expression of PD-1, PD-L1 and tumor-infiltrating lymphocytes (TILs) of primary CM using immunofluorescence staining in **Chapter 5**. The results show that PD-L1 is expressed in some CM, and this expression is correlated with distant metastases and a worse melanoma-related survival. Large tumors contain a lower density of TILs

than small tumors. Our explorative study indicates that immunotherapy targeting the PD-1/PD-L1 axis should be considered for patients with CM.

In **Chapter 6**, we reveal that downregulation of HLA class I expression occurs frequently in CM, and thick tumors show a low HLA class I expression. Moreover, HLA class I expression is not correlated with PD-L1/PD-1 status, but respectively associates and tends to associate with macrophages and $CD8^+$ T cells, which produce interferon gamma, an essential cytokine for HLA class I regulation. Therefore, to mimic the tumor environment, CM cell lines were incubated with interferon gamma, which indeed led to elevation of mRNA and protein expression of HLA class I. Thick tumors that lack HLA class I may be inappropriate targets for T cell mediated therapies, unless cytokines are added that will upregulate their expression.

Finally, in **Chapter 7** we summarize and discuss the conclusions according to the studies presented in the thesis.

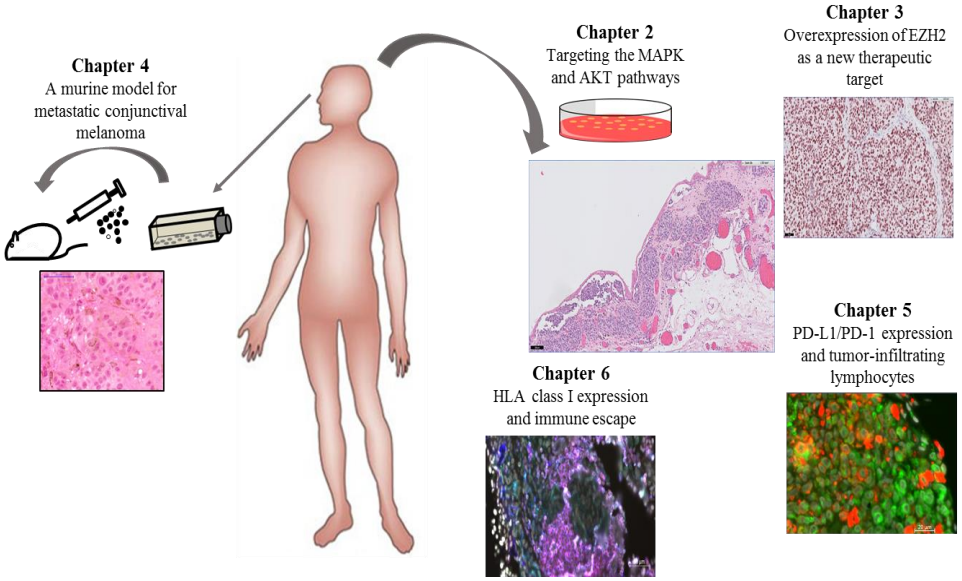

Figure 4. Overview of different chapters.

References

[1] Cramer, SF. The origin of epidermal melanocytes. Implications for the histogenesis of nevi and melanomas. Arch Pathol Lab Med. 1991;115:115-119.

[2] McLaughlin CC, Wu XC, Jemal A, Martin HJ, Roche LM, Chen VW. Incidence of noncutaneous melanomas in the U. S. Cancer. 2005;103:1000–1007.

[3] Isager P, Engholm G, Overgaard J, Storm H. Uveal and conjunctival malignant melanoma in denmark 1943-97: observed and relative survival of patients followed through 2002. Ophthalmic Epidemiol. 2006;13:85–96.

[4] Taban M, Traboulsi EI. Malignant melanoma of the conjunctiva in children: a review of the international literature 1965-2006. J Pediatr Ophthalmol Strabismus. 2007;44:277-82; quiz 298-9.

[5] Tuomaala S, Eskelin S, Tarkkanen A, Kivelä T. Populationbased assessment of clinical characteristics predicting outcome of conjunctival melanoma in whites. Invest Ophthalmol Vis Sci. 2002;43:3399-3408.

[6] Yu GP, Hu DN, McCormick S, Finger PT. Conjunctival melanoma: is it increasing in the United States? Am J Ophthalmol. 2003; 135:800-806.

[7] Triay E, Bergman L, Nilsson B, All-Ericsson C, Seregard S. Time trends in the incidence of conjunctival melanoma in Sweden. Br J Ophthalmol. 2009; 93:1524–1528.

[8] Shields CL, Markowitz JS, Belinsky I, Schwartzstein H, George NS, Lally SE, Mashayekhi A, Shields JA. Conjunctival melanoma: outcomes based on tumor origin in 382 consecutive cases. Ophthalmology. 2011;118:389-395.e1-2.

[9] Missotten GS, Keijser S, De Keizer RJ, De Wolff-Rouendaal D. Conjunctival melanoma in the Netherlands: a nationwide study. Invest Ophthalmol Vis Sci. 2005; 46:75-82.

[10] Wenkel H, Rummelt V, Naumann GO. Malignant melanoma of the conjunctiva with intraocular extension. Arch Ophthalmol. 2000;118:557-560.

[11] Schlereth SL, Iden S, Mescher M, Ksander BR, Bosch JJ, Cursiefen C, Heindl LM. A novel model of metastatic conjunctival melanoma in immune-competent mice. Invest Ophthalmol Vis Sci. 2015;56:5965-5973.

[12] Shields CL, Shields JA, Gunduz K, Cater J, Mercado GV, Gross N, et al. Conjunctival melanoma: risk factors for recurrence, exenteration, metastasis, and death in 150 consecutive patients. Archives of ophthalmology. 2000;118:1497-507.

[13] Kujala E, Tuomaala S, Eskelin S, Kivelä T. Mortality after uveal and conjunctival melanoma: which tumour is more deadly? Acta Ophthalmol. 2009;87(2):149-53.

[14] Shildkrot Y, Wilson MW. Conjunctival melanoma: pitfalls and dilemmas in management. Curr Opin Ophthalmol. 2010;21:380–386.

[15] Cao J, Heijkants RC, Jochemsen AG, Dogrusöz M, de Lange MJ, van der Velden PA, van der Burg SH, Jager MJ, Verdijk RM. Targeting of the MAPK and AKT pathways in conjunctival melanoma shows potential synergy. Oncotarget. 2016. doi: 10.18632/oncotarget.10770.

[16] Jakobiec FA, Folberg R, Iwamoto T. Clinicopathologic characteristics of premalignant and malignant melanocytic lesions of the conjunctiva. Ophthalmology. 1989;96:147-166.

[17] Shields JA, Shields CL, Mashayekhi A, Marr BP, Benavides R, Thangappan A, Phan L, Eagle RC Jr. Primary acquired melanosis of the conjunctiva. Risks for progression to melanoma in

311 eyes. The 2006 Lorenz E. Zimmerman Lecture. Ophthalmology. 2008;115:511-519.

[18] Shields JA, Shields CL, Mashayekhi A, Marr BP, Benavides R, Thangappan A, Phan L, Eagle RC Jr. Primary acquired melanosis of the conjunctiva: experience with 311 eyes. Trans Am Ophthalmol Soc 2007;105:61-71.

[19] Paridaens AD, Minassian DC, McCartney AC, Hungerford JL. Prognostic factors in primary malignant melanoma of the conjunctiva: a clinicopathological study of 256 cases. Br J Ophthalmol 1994;78:252–259.

[20] Werschnik C, Lommatzsch PK. Long-term follow-up of patients with conjunctival melanoma. Am J Clin Oncol 2002;25:248–255.

[21] Yousef YA, Finger PT. Predictive Value of the Seventh Edition American Joint Committee on Cancer Staging System for Conjunctival Melanoma AJCC Staging for Conjunctival Melanoma. Arch Ophthalmol 2012;130:599-606.

[22] Tuomaala S, Kivela T. Metastatic pattern and survival in disseminated conjunctival melanoma: implications for sentinel lymph node biopsy. Ophthalmology 2004;111:816–821.

[23] Savar A, Ross MI, Prieto VG, Ivan D, Kim S, Esmaeli B. Sentinel lymph node biopsy for ocular adnexal melanoma: experience in 30 patients. Ophthalmology 2009;116:2217-2223.

[24] Heindl LM, Hofmann-Rummelt C, Adler W, Bosch JJ, Holbach LM, Naumann GO, Kruse FE, Cursiefen C. Prognostic significance of tumor-associated lymphangiogenesis in malignant melanomas of the conjunctiva. Ophthalmology 2011;118:2351-2360.

[25] Gloor P, Alexandrakis G. Clinical characterization of primary acquired melanosis. Invest Ophthalmol Vis Sci. 1995;36:1721-1729.

[26] Pe'er J, Frucht-Pery J. The treatment of primary acquired melanosis (PAM) with atypia by topical Mitomycin C. Am J Ophthalmol 2005;139:229–234.

[27] Finger PT, Sedeek RW, Chin KJ. Topical interferon alfa in the treatment of conjunctival melanoma and primary acquired melanosis complex. Am J Ophthalmol 2008;145:124–129.

[28] Herold TR, Hintschich C. Interferon alpha for the treatment of melanocytic conjunctival lesions. Graefes Arch Clin Exp Ophthalmol. 2010;248:111-115.

[29] Lim LA, Madigan MC, Conway RM. Conjunctival melanoma: a review of conceptual and treatment advances. Clin Ophthalmol. 2013;6:521-531.

[30] Damato B, Coupland SE. An audit of conjunctival melanoma treatment in Liverpool. Eye (Lond). 2009;23:801-809.

[31] Damato B, Coupland SE. Management of conjunctival melanoma. Expert Rev Anticancer Ther. 2009;9:1227-1239.

[32] Kao A, Afshar A, Bloomer M, Damato B. Management of primary acquired melanosis, nevus, and conjunctival melanoma. Cancer Control. 2016;23:117-125.

[33] Finger PT, Czechonska G, Liarikos S. Topical mitomycin C chemotherapy for conjunctival melanoma and PAM with atypia. Br J Ophthalmol. 1998;82:476-479.

[34] Ditta LC, Shildkrot Y, Wilson MW. Outcomes in 15 patients with conjunctival melanoma treated with adjuvant topical mitomycin C: complications and recurrences. Ophthalmology. 2011;118:1754-1759.

[35] Demirci H, McCormick SA, Finger PT. Topical mitomycin chemotherapy for conjunctival malignant melanoma and primary acquired melanosis with atypia: clinical experience with histopathologic observations. Arch Ophthalmol. 2000;118:885-891.

[36] Kurli M, Finger PT. Topical mitomycin chemotherapy for conjunctival malignant melanoma and primary acquired melanosis with atypia: 12 years' experience. Graefes Arch Clin Exp Ophthalmol. 2005;243:1108-1114.

[37] Wuestemeyer H, Sauerwein W, Meller D, Chauvel P, Schueler A, Steuhl KP, Bornfeld N, Anastassiou G. Proton radiotherapy as an alternative to exenteration in the management of extended conjunctival melanoma. Graefes Arch Clin Exp Ophthalmol. 2006;244:438-446.

[38] de Waard NE, Cao J, McGuire SP, Kolovou PE, Jordanova ES, Ksander BR, Jager MJ. A murine model for metastatic conjunctival melanoma. Invest Ophthalmol Vis Sci. 2015;56:2325-2333.

[39] Folberg R, Baron J, Reeves RD, Stevens RH, Bogh LD. Animal model of conjunctival primary acquired melanosis. Ophthalmology. 1989;96:1006-1013.

[40] Griewank KG, Westekemper H, Murali R, et al. Conjunctival melanomas harbor BRAF and NRAS mutations and copy number changes similar to cutaneous and mucosal melanomas. Clin Cancer Res. 2013;19:3143-3152.

[41] Gear H, Williams H, Kemp EG, Roberts F. BRAF mutations in conjunctival melanoma. Invest Ophthalmol Vis Sci. 2004; 45:2484-2488.

[42] Spendlove HE, Damato BE, Humphreys J, Barker KT, Hiscott PS, Houlston RS. BRAF mutations are detectable in conjunctival but not uveal melanomas. Melanoma Res. 2004; 14:449-452.

[43] Alessandrini L, Parrozzani R, Bertorelle R, Valentini E, Candiotto C, Giacomelli L, Midena E, Blandamura S. C-Kit SCF receptor (CD117) expression and KIT gene mutation in conjunctival pigmented lesions. Acta Ophthalmol. 2013;91:e641-e645.

[44] Zoroquiain P, Fernandes BF, Gonzalez S, Novais GN, Schalper KA, Burnier MN Jr. p16ink4a expression in benign and malignant melanocytic conjunctival lesions. Int J Surg Pathol. 2012;20:240-245.

[45] Vajdic CM, Hutchins AM, Kricker A, Aitken JF, Armstrong BK, Hayward NK, Armes JE. Chromosomal gains and losses in ocular melanoma detected by comparative genomic hybridization in an Australian population-based study. Cancer Genet Cytogenet. 2003;144:12-17.

[46] Lake SL, Jmor F, Dopierala J, Taktak AF, Coupland SE, Damato BE. Multiplex ligation-dependent probe amplification of conjunctival melanoma reveals common BRAF V600E gene mutation and gene copy number changes. Invest Ophthalmol Vis Sci. 2011;52:5598-5604.

[47] Busam KJ, Fang Y, Jhanwar SC, Pulitzer MP, Marr B, Abramson DH. Distinction of conjunctival melanocytic nevi from melanomas by fluorescence in situ hybridization. J Cutan Pathol. 2010;37:196-203.

[48] Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients. JAMA. 1998; 279:1200-1205.

[49] Cheng Y, Zhang G, Li G. Targeting MAPK pathway in melanoma therapy. Cancer Metastasis Rev. 2013;32:567-584.

[50] Pahlitzsch M, Mai C. Conjunctival melanoma and BRAF inhibitor therapy. J Clin Exp Ophthalmol. 2014;5:322.

[51] A. Maleka, G. Åström, P. Byström, G. J. Ullenhag. A case report of a patient with metastatic ocular melanoma who experienced a response to treatment with the BRAF inhibitor vemurafenib. BMC Cancer. 2016;16:634.

[52] Weber JL, Smalley KS, Sondak VK, Gibney GT. Conjunctival melanomas harbor BRAF and NRAS mutations--Letter. Clin Cancer Res. 2013;19:6329-6330.

[53] Handy DE, Castro R, Loscalzo J. Epigenetic modifications: basic mechanisms and role in cardiovascular disease. Circulation. 2011;123:2145-2156.

[54] Ciferri C, Lander GC, Maiolica A, Herzog F, Aebersold R, Nogales E. Molecular architecture of human polycomb repressive compex 2. eLife. 2012;1:e00005. doi: 10.7554/eLife.00005.

[55] Riquelme E, Behrens C, Lin HY, Simon G, Papadimitrakopoulou V, Izzo J, Moran C, Kalhor N, Lee JJ, Minna JD, Wistuba II. Modulation of EZH2 expression by MEK-ERK or PI3K-AKT signaling in lung cancer is dictated by different KRAS oncogene mutations. Cancer Res. 2016;76:675-685.

[56] Morin RD, Johnson NA, Severson TM, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat Genet. 2010; 42:181–185.

[57] Tiffen J, Gallagher SJ, Hersey P. EZH2: an emerging role in melanoma biology and strategies for targeted therapy. Pigment Cell Melanoma Res. 2015;28:21-30.

[58] Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, Salvesen HB, Otte AP, Akslen LA. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol. 2006;24:268-273.

[59] Kim KH, Roberts CW. Targeting EZH2 in cancer. Nat Med. 2016;22:128-134.

[60] Becker JC, Terheyden P, Kämpgen E, Wagner S, Neumann C, Schadendorf D, Steinmann A, Wittenberg G, Lieb W, Bröcker EB. Treatment of disseminated ocular melanoma with sequential fotemustine, interferon alpha, and interleukin 2. Br J Cancer. 2002;87:840-845.

[61] Weber JS, D'Angelo SP, Minor D, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. 2015;16:375-384.

[62] Mahoney KM, Freeman GJ, McDermott DF. The next immune checkpoint inhibitors: PD-1/PD-L1 blockade in melanoma. Clinical Ther. 2015;37:765-782.

[63] Ford J, Thuro BA, Thakar S, Hwu WJ, Richani K, Esmaeli B. Immune checkpoint inhibitors for treatment of metastatic melanoma of the orbit and ocular adnexa. Ophthal Plast Reconstr Surg. 2016. doi: 10.1097/IOP.0000000000000790.

[64] Speetjens FM, de Bruin EC, Morreau H, Zeestraten EC, Putter H, van Krieken JH, van Buren MM, van Velzen M, Dekker-Ensink NG, van de Velde CJ, Kuppen PJ. Clinical impact of HLA class I expression in rectal cancer. Cancer Immunol Immunother. 2008;57:601-609.

[65] Ericsson C, Seregard S, Bartolazzi A, Levitskaya E, Ferrone S, Kiessling R, Larsson O. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Invest Ophthalmol Vis Sci. 2001;42:2153-2156.

[66] Jager MJ, Hurks HM, Levitskaya J, Kiessling R. HLA expression in uveal melanoma: there is no rule without some exception. Hum Immunol. 2002;63:444-451.

[67] Westekemper H, Karimi S, Süsskind D, Anastassiou G, Freistühler M, Meller D, Zeschnigk M, Steuhl KP, Bornfeld N, Schmid KW, Grabellus F. Expression of MCSP and PRAME in conjunctival melanoma. Br J Ophthalmol. 2010;94:1322-1327.

[68] Carreno BM, Magrini V, Becker-Hapak M, Kaabinejadian S, Hundal J, Petti AA, Ly A, Lie WR, Hildebrand WH, Mardis ER, Linette GP. Cancer immunotherapy. A dendritic cell

vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. Science. 2015;348:803-808.

[69] Garg AD, Coulie PG, Van den Eynde BJ, Agostinis P. Integrating next-generation dendritic cell vaccines into the current cancer immunotherapy landscape. Trends Immunol. 2017. doi: 10.1016/j.it.2017.05.006.

[70] Dunn J, Rao S. Epigenetics and immunotherapy: The current state of play. Mol Immunol. 2017;87:227-239.