Regulation of inflammation in uveal melanoma

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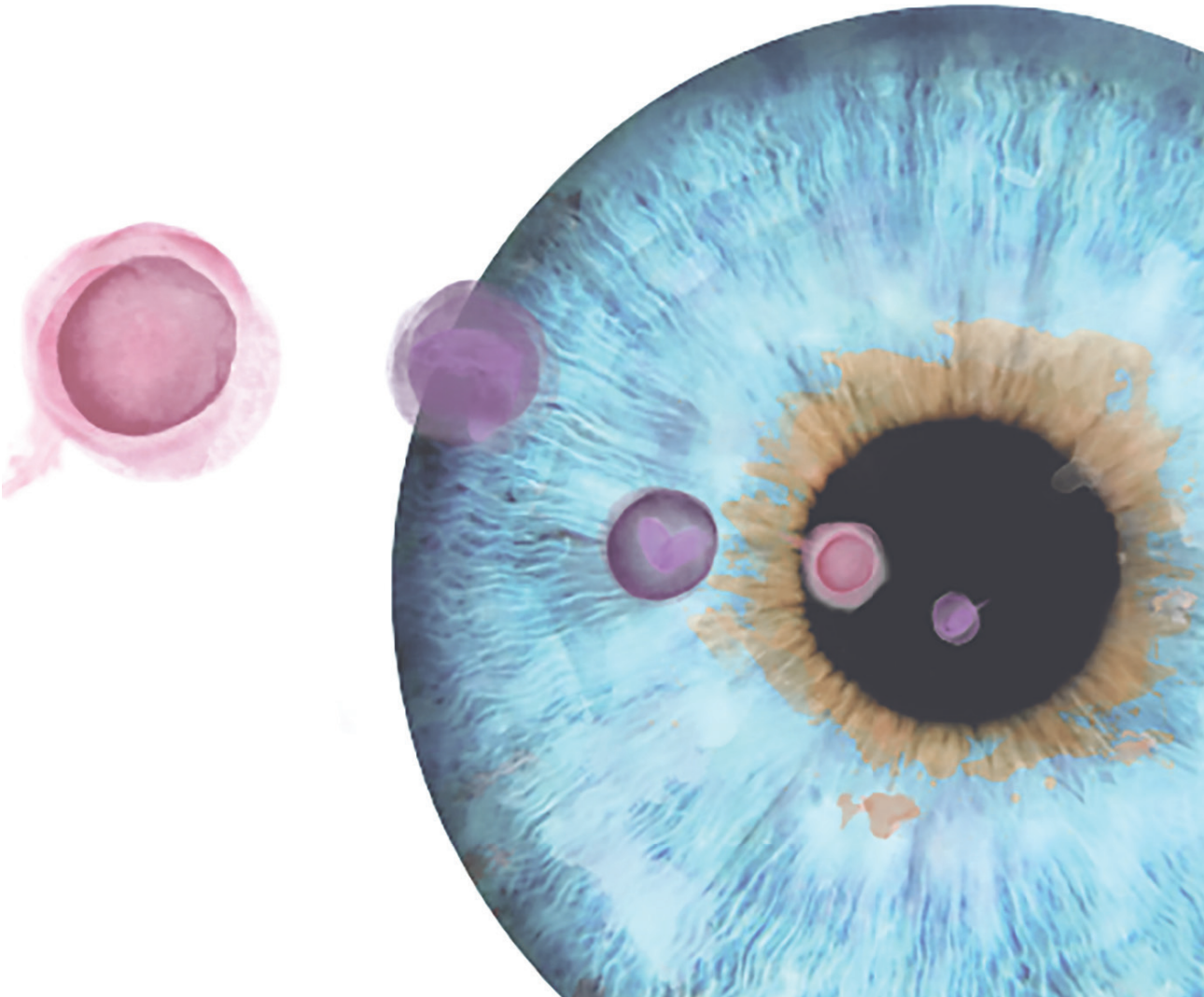
Chapter 1

Introduction and outline of thesis



Introduction

Uveal melanoma

Uveal melanoma (UM) is the most common intraocular malignancy in adults. This disease mainly occurs in Caucasians with a light skin, blond hair and blue eyes and has an incidence rate of 7 per million per year (ranges from 4 to 11) in northern Europe and America (Metzelaar-Blok 2001, Virgili 2007, Singh 2003, Singh 2011, Houtzagers 2020). The mean age at diagnosis is 60 years (Weis 2006, Singh 2011). Symptoms include blurred, distorted vision and/or pain in the eye, although approximately one third of patients have been reported as asymptomatic (Damato 2012). The delayed detection causes the patient to carry a more advanced tumour which unfortunately increases the chance of losing the eye (Dogrusoz 2017).

Treatment options for the primary tumour which aim to preserve the eye include plaque brachytherapy, proton beam therapy and stereotactic radiation (Jager 2020), but if the treatment does not show any benefit mostly due to large tumour size, then removal of the globe is advised.

Up to 50% of the patients develop metastases mostly targeting the liver; once detected, the median survival is approximately 4-12 months (Augsburger 2008). As there are no curative treatment options available for the metastases in UM, no improvement in survival has been observed during the last fifty years (Roelofsen 2021). The tumour arises from the uveal tract and involves the pigmented tissues, including the iris, ciliary body and the choroid (Figure 1). 86 percent of the cases arise from the choroid (Mclaughlin 2005). For prognosis, the best location is the iris, mainly because of early detection and small tumour size (Shields 2009) while the worst is known to be the ciliary body, which has the highest chance of metastasis because of a larger tumour size (Jager 2020).

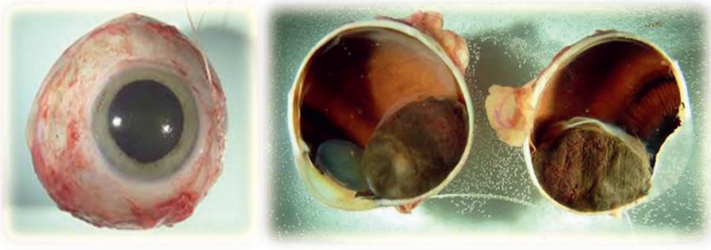


Figure 1: An eye involved with choroidal melanoma

UM are mostly composed of two types of cells, epithelioid and spindle cells. Epithelioid cells are large with large nuclei and round nucleoli located inside a high amount of cytoplasm while spindle cells are small with a tight extracellular space, small nuclei and nucleoli and less cytoplasm (Myamoto 2012). Tumours with an epithelioid cell type are often associated with metastasis (Callender 1931). Tumour basal diameter and thickness are other characteristics known to be associated with a higher melanoma-related mortality (Kujala 2003) and increased risk for metastasis (Shields 2009).

Another way of classification is the Tumour Node Metastasis (TNM) staging, which is based on tumour size, involvement of the ciliary body and extrascleral extension (Edge 2010). This is now known as the American Joint Committee on Cancer (AJCC) classification (Kivela 2013).

Studies have reported that older age at diagnosis is associated with worse prognosis in UM, exerting the highest metastatic risk for ages above 60 years old (Shields 2012). Gender is not highly associated although a study reported that the tendency toward development of melanoma-related metastasis is higher in males compared to females (Zloto 2013).

Uveal melanoma chromosome aberrations

Different genetic abnormalities are involved in the creation of this rare and invasive ocular malignancy. The different chromosomal aberrations which are mostly observed include loss of chromosome 1p, 3, 6q and gain in 6p or 8q (Prescher 1992, White 1998, Kilic 2005, Damato 2009, Dogrosuz 2018, Shields 2017). Loss of one copy of chromosome 3 (M3) is the most reliable biomarker for patient survival. M3 is considered as an early copy number change in UM and has been associated with different histopathological characteristics such as largest basal tumor diameter (Kiliç 2006) and epithelioid cell type (Scholes 2003).

Other chromosome abnormalities enhancing the disease include extra copies of chromosome 8q. Chromosome 8q has been known as an important location responsible for the attraction of macrophages to the eye which could contribute to the development of metastasis in UM (De Lange 2015, Versluis 2015) and often coincides with M3 (Sisley 1997). The combination of loss of chromosome 3 and gain in 8q strengthens the progression of this disease and increases the risk of developing metastasis (Cassoux 2014, Versluis 2015, Gezgin 2017).

Uveal melanoma somatic mutations

Somatic genetic mutations play a major role in the development of uveal melanoma. Two very early somatic mutations give the melanocyte the ability to transform into a uveal nevus: these are GNAQ or GNA11 gene mutations (Vader 2017). These mutations are very common in UM (Van Raamsdonk 2009, van Raamsdonk 2010; de Lange 2015; Piaggio 2019). GNAQ and GNA11 switch on G protein signaling by activating inositol-3-phosphate, diacylglycerol, and cyclic AMP and then activate a set of signaling pathways important for cell growth and proliferation (O'Hayre 2014).

Mutations in the BRCA1 associated protein-1 (BAP1) gene may subsequently occur. The BAP1 gene is located on chromosome 3p21.1 and codes for a ubiquitin carboxy-terminal hydrolase (UCH). Like in other cancers (Jensen 1998), BAP1 acts as a tumour suppressor in UM as well (Harbour 2010), and lack of expression is directly associated with the development of metastasis (Harbour 2010, van Essen 2014, Koopmans 2014).

UM with a high risk of metastases have lower level of BAP1 expression compared to low risk tumours (Harbour 2010, Robertson 2017). Loss of BAP1 expression leads to the attraction of lymphocytes inside the eye, which increases the local inflammation (Gezgin 2017).

Uveal melanoma Immunology

The Immune-privileged eye

Immune-privilege is an adaptive mechanism that occurs in certain organs in order to avoid damages by the immune system. One such organ is the eye, where many cells lack regenerative activity. Suppressing inflammation is important to maintain visual clarity and retinal function. To be able to reach these goals the eye uses a set of different mechanisms: the internal part of the globe is filled with liquids namely the aqueous humor and vitreous fluid, which contain immune suppressive factors such as neuropeptides, αMSH (melanocyte stimulating hormone) (Stein 2007), cytokines such as TGFβ-2, complement inhibitors and macrophage inhibitory factor (MIF) (Apte 1998).

Another mechanism for immunosuppression in the eye is the ability of some local cells such as corneal endothelial cells to transform active T cells into regulatory T cells (Treg cells), thus lowering their cytotoxic activity (Yamada 2010). In the back of the eye, retinal pigment epithelial (RPE) cells form tight junctions and limit the local entrance of leukocytes into the eye

(Sugita 2009). Pigmented epithelial (PE) cells of the iris also inhibit T cell activity through CTLA-4 binding (Sugita 2003) while retinal and ciliary body PE cells suppress T cell activity by producing TGF-β1/2 (Sugita 2006).

Antigens present in the anterior chamber of murine eyes showed ability to escape from the orbit and activate the host immune system; however, the anterior chamber route led to an inhibited immune response to the intraocular antigens, and helped to preserve retinal function; this phenomenon is known as the anterior chamber associated immune deviation (ACAID) (Streilein 1996).

The inflammatory phenotype in UM

Inflammation is known as the seventh hallmark of cancer (Collota 2009). Based on the type of involved immune cells, inflammation can either act as a growth initiator or inhibitor in a malignancy. In cancers such as cutaneous melanoma, breast carcinoma or non-Hodgkin lymphoma, it has been fully accepted that the recruitment of immune cells into the tumour loci is beneficiary for the patient's overall survival (Mlecnik 2011) while this is not the case for UM.

In UM, immune cells are often recruited to the tumour site. This was already found in early studies: infiltrating lymphocytes (CD3, CD4, and CD8) and macrophages (CD11, CD68, and CD163) were described (De la Cruz 1990, Makitie 2001, Meecham 1992, Tobal 1993, De Waard-Siebinga 1996).

Extensive infiltration of lymphocytes and macrophages is associated with poor prognosis in UM (De Lange 2015, Robertson 2017). The accumulation of these cells is positively associated with loss of one copy of chromosome 3 and high levels of expression of HLA Class I and Class II (Maat 2008; de Waard-Siebinga 1996, Jager 2002).

The high number of infiltrating leukocytes and high expression of HLA forms the inflammatory phenotype which is a strong marker for the development of metastasis and negatively impacts survival (Maat 2008, Bronkhorst 2011, and Gezgin 2017).

NK cells in UM

NK cells belong to the innate immune system and are present in UM in a low percentage (Ksander 1991). These cells are recognized by the presence of CD56 marker and absence of CD3. Because of the presence of two strong inhibitors TGF-β and MIF, inside the aqueous humor, NK cells are not able to take any action and are kept inactive in the eye (Apte 1996).

NK-cell mediated lysis is inversely correlated with the level of HLA Class I expression on tumour cells (Ma 1995). Normal eye tissues express low levels of HLA (Niederkorn 2012), making them a target for NK cells. The high level of expression of HLA Class I in most high risk UM provides these tumour cells with an escape mechanism that allows spread through the bloodstream to distant organs such as the liver.

Macrophages in UM

Macrophages are components of the innate immune system involved in host defense mechanisms and are derived from differentiated circulating monocytes. These cells can become activated by Th1 and Th2 cells; based on the type of cytokines they receive, they become polarized into two different subsets of cells, namely M1 and M2 with different programs (Mills 2000). These two types of macrophages act in opposite ways.

The M1 macrophages are also known as classical macrophages and are initiators of anti-tumour responses; they express high levels of HLA Class II. M1 macrophages are mainly involved in antibacterial and anti-angiogenic functions and are able to produce nitric oxide (NO), IL-12 and TNF.

The M2 subtype has a low anti-tumour activity and has a low HLA Class II expression. M2 macrophages are involved in anti-inflammatory and proangiogenic activity and mainly produce IL-10, L-1ra, and type II IL-1 decoy receptor (Mills 2000, Mantovani 2002).

One important underlying mechanism for the recruitment of macrophages towards the tumour site is the presence of inflammatory chemoattractants; CCL2, CXCL12, CXCL8, CXCL1, CXCL13, CCL5 (Nesbit 2002, Azenshtein 2002) attract macrophages and thereby further induce inflammation and angiogenesis at the tumour site. The presence of macrophages has been reported to be positively correlated with the presence of T cells in UM (Bronkhorst 2012, De Lange 2018). The most dominant macrophages in UM are the $CD68^{+}CD163^{+}$ M2 subtype. A high density of these cells is strongly associated with M3 in UM (Bronkhorst 2011). Macrophage density is positively associated to risk factors in uveal melanoma. High numbers of macrophages correlate with more epithelioid cells, high pigmentation and a high vascular density in the tumour area. Moreover, macrophage infiltration correlates with higher rate of metastasis and a shorted survival in UM patients (Makitie 2001).

T cells in UM

T lymphocytes belong to the adaptive immune response. These cells are derived from hematopoietic stem cells in the bone marrow and become mature and differentiate into subtypes inside the thymus: CD8 cytotoxic T cells, CD4 helper cells (Th1, Th2, and Th17) and regulatory T cells. CD8 T cells are the main cells in eliminating invaders with cytotoxic activity. These cells recognize foreign peptides as presented by HLA Class I using their TCR receptor and, following secretion of cytotoxic cytokines, recruit other immune cells in order to kill the pathogens. CD4 helper cells act to mount an immune response indirectly through the secretion of inflammatory factors and are even able to suppress immune responses (Biswas 2010). The regulatory T cells are important in controlling how the immune system acts in response to self-antigens and therefore are often considered as suppressor T cells able to protect self-cells from autoimmune reactions.

The presence of T cells has different roles in different cancers (Yu 2006). For example, in cutaneous melanoma, the presence of T cells improves survival (Mihm 2015); by contrast, T cell infiltration contributes to a worse survival in UM (de la Cruz PO 1990, Whelchel 1993). One reasons behind the aforementioned phenomenon is the presence of Tregs in UM tumours (Lagouros 2009, Bronkhorst 2012). The tumour is able to induce a set of Treg cells (CD4) which may suppress local anti tumoural activity.

HLA Expression in UM

HLA expression is an important inflammatory marker in UM. Based on their structure and type of interaction, HLA molecules are categorized into two types.

HLA Class I molecules consist of classic HLA-A, -B and -C and non-classic HLA-E, -L, -J, -K, -H, and –G subtypes (Goldsby 2003). Classic HLA Class I are expressed on almost all nucleated cells and present antigens to T cell receptors (TCR) of CD8 T cells (Szeto 2021). They are made of one heavy polypeptide chain which is non-covalently associated with β2-microglobulin (β2-m).

The second type, HLA Class II, includes HLA-DP, DM, DO, DQ and –DR (Goldsby 2003) which are only expressed on specific immune cells such as antigen presenting cells (monocytes, macrophages and dendritic cells), thymic epithelium cells and activated T cells. These molecules bind to CD4 T cells and can start immune responses against foreign peptides (Roche 2015).

The natural expectation is that tumour cells decrease their surface HLA expression in order to avoid recognition by cytotoxic T cells (Garrido 1993), whereas the opposite occurs in UM (Jager 2002): as tumour spread from the eye takes place, tumour cells might encounter NK cells present in the blood which target cells with a low HLA Class I expression (Ma 1995). The finding that higher levels of HLA Class I, HLA Class II and β2-microglobulin expression are associated with a lower survival supports this idea (Blom 1997; Erisson 2001).

On the other hand, a high expression of HLA Class I antigens may allow an effective T cell response once metastases have reached the liver. One reason for ineffective local T cell responses might be the loss of expression of specific alleles, thereby decreasing the sensitivity to cytotoxic T cell-mediated lysis, even when high levels of other alleles are present. HLA allotype loss is another strategy which occurs in both HLA-A and HLA-B and might exhibit as haplotype loss or complete loss (Hurks 2000, Anastassiou 2003). An analysis of a series of cell lines showed deficiencies of several alleles. Even after exposure of the cell lines to inflammatory cytokines such as IFNγ and IFNα, some alleles did not show expression (de Waard-Siebinga 1995).

Potential inflammatory regulators in UM

As already mentioned, the presence of an inflammatory phenotype is linked to specific chromosome copy number aberrations, especially with monosomy 3/loss of BAP1 expression (Maat 2008, Bronkhorst 2012, Robertson 2017, Gezgin 2017). The inflammatory phenotype is characterized by an increased density of infiltrating lymphocytes and macrophages, and an increased expression of HLA Class I and II antigens. We set out to study which pathways may play a role in the regulation of this inflammatory phenotype. We looked at the NFkB pathway, epigenetic regulators such as miRNAs and HDACs, and at the potential influence of immune checkpoint molecules.

NFkB pathway as a mediator between inflammation and cancer

An important signaling pathway which regulates the immune system, involved in inflammatory processes is the NFkB pathway. After activation, this pathway ultimately leads to the production of inflammatory cytokines and regulates immune cell activation (Lawrence 2009, Oh 2013). Multiple studies have reported the upregulation of the NFkB pathway in several type of cancers giving it a role in uncontrolled proliferation, angiogenesis, metastasis and resistance to different therapies (Pires 2018). It has been reported that the NFkB pathway is involved in the progression of uveal melanoma (Mier 2007, Dror 2009). Until now no studies have looked at the relationship between this pathway and the inflammatory phenotype of UM.

Epigenetics and regulation of inflammation

Epigenetics is the phenomenon which regulates gene expression without altering the gene sequence. One approach is through methylation of promoters, which represses transcription. Another set of epigenetic enzymes which alter gene expression in malignancies are histone deacetylases (HDAC). These enzymes repress gene expression by the removal of acetyl groups from the histone tails, compacting the chromatin structure and making the promoter unreachable. HDACs are often overexpressed in tumours which potentially enhance the proliferation and development of cancer cells by repressing the expression of cell cycle inhibitors (Halkidou 2004, Song 2005).

MiRNAs as regulators of inflammation

A group of non-translated molecules referred to as miRNAs are involved in the epigenetic regulation of gene expression. These short RNAs are often 17-22 base pairs long and bind to specific mRNA based on complementary sequencing. By binding to 3´UTR regions of complimentary mRNAs, they either stimulate or delay translation of specific transcribed genes (Wahid, 2001). Recent studies have found roles for miRNAs in the invasive behavior of tumour cells. MiRNAs could target oncogenes, tumour suppressors, modulate stem cell characteristics, alter epithelial mesenchymal transition ability, influence cells residing in the tumour microenvironment and therefore play a role in the development of metastasis (as reviewed by Kim 2019).

Microarray studies on different types of tumours indicate that in addition to a tumour suppressive role, miRNAs could also act as oncogenes and halt tumour suppressor genes at a post-transcriptional level; for example the effect of mir-106a on Rb in colon cancer and mir-20a on TGFBR2 in lung cancer (Volinia 2006). In addition, inflammation could also affect miRNA expression in cancers (Wu 2008)

Different studies report different miRNAs to be either up or downregulated in high risk UM tumours (Worley 2008, Smit 2019, Augton 2020) but none of them have studied the involvement of miRNAs in the inflammatory processes of this disease.

Immune checkpoints as Immune modulators

The most abundant immune cells present in the UM tumour niche are T cells; while these cells form extensive colonies around the malignant cells, they are mostly not effective in destroying them and are considered “exhausted” (Niederkorn 2009). The exact reason for this phenomenon is still unknown but suggests a possible role for immune checkpoints.

Well known immune checkpoints are CTLA-4 and PD-L1. CTLA-4 is one of the checkpoints expressed on effector and regulatory T cells which is very similar to CD28 and therefore is able to bind to CD80/86 with higher affinity and hence reduce its stimulatory effect (Algere 2001).

PD-1, which is mainly expressed on T cells and B cells. PD-1 binds to its ligands PD-L1 and PD-L2, which are expressed on the cell surface of tumour cells (Topalian 2012).

Despite several attempts toward the use of checkpoint therapy in UM, most have not been successful enough to serve as an appropriate adjuvant therapeutic approach for the treatment of UM metastasis (Rodrigues 2019); recently, research pointed to another immune checkpoint, Lymphocyte Activation Gene-3 (LAG3) (Durante 2020).

LAG3 is naturally present on the surface of T and NK cells and is relevant for the prevention of auto-immunity (Triebel 1990). The canonical ligand for LAG3 is HLA Class II. HLA Class II is believed to be associated with the development of metastasis in UM (Krishnakumar 2003). In 1988 Jager et al reported that HLA-DQ was associated with infiltration in UM (Jager 1988).

Taken together it seems that the high LAG3 /HLA Class II expression detected in UM tumours might serve as an appropriate mechanism for reviving the cytotoxic anti-tumour ability of CD8 cells.

Outline of thesis

As HLA Class I expression is an important target for cytotoxic T cells but an in inhibitor of NK cells, we were interested in the regulation of its expression. It is furthermore a prognostic marker, associated with loss of one copy of chromosome 3 and loss of BAP1 expression. In **chapter 2,** we review HLA expression in UM, how it is involved in the inflammatory phenotype, how it is regulated and how putative treatments might be effective in its expression. In addition, we report results of experiments with regard to HLA Class I expression in UM cell lines: we show that allelic defects are present in the cell lines even after IFNɤ stimulation. We report how different drugs impact HLA Class I expression both in a negative and positive manner.

In **chapter 3,** we investigate the potential role of the NFkB pathway in the regulation of inflammation in UM and its potential association with HLA Class I expression. We wonder whether the expression of NFkB components are related to the tumours chromosome 3/BAP1 status. We report that members of both canonical and non-canonical NFkB pathway are related to

high amounts of infiltrating T cells and macrophages and also show an association with loss of chromosome3/BAP1 in UM.

In order to increase our understanding for the reason behind the elevated HLA Class I expression in UM tumours, we investigate the involvement of epigenetics in **chapter 4**. We focus on a set of epigenetic enzymes called histone deacetylases and report that these regulators are highly expressed in Monosomy 3 UM. Moreover we show that the chemotherapeutic HDAC inhibitor, Quisinostat, is not only able to inhibit cell growth in-vitro but further increases HLA Class I at both the mRNA and protein level and hence might impact immunotherapeutic approaches when used as adjuvant therapy.

In **chapter 5** we wonder whether HDAC expression is influenced by the presence of infiltrating lymphocytes and macrophages. We find that HDAC expression is positively correlated with the presence of infiltrating immune cells; we show that HDACs are induced by IFNɣ and therefore suggest a role for infiltrating cells in the regulation of HDAC enzymes.

In **chapter 6** we focus on miRNA's as another set of epigenetic regulators of inflammation. We investigate the potential relation of a set of 125 miRNA's with HLA Class I expression and the presence of an infiltrate in UM and report two patterns of miRNA expression.

In **chapter 7** we study the LAG3 immune checkpoint in UM tumours. As immune checkpoints might be responsible for the T cell exhaustion which is observed in UM, we investigate the involvement of LAG in prognostication and study how LAG3 and its ligands are distributed among different UM tumours in order to increase knowledge towards the design of new adjuvant therapy for the treatment of UM metastasis.

Finally in **chapter 8,** we summarize the different chapters and report our general conclusion based on what we have understood from the findings of this thesis.

References

1. Metzelaar-Blok, J.A.W.; Ter Huurne, J.A.C.; Hurks, H.M.H.; et al. Characterization of Melanocortin-1 Receptor gene variants in uveal melanoma patients. Invest. Ophthalmol. Vis. Sci. 2001, 42, 1951-1954.
2. Virgili, G.; Gatta, G.; Ciccolallo, L.; et al. EUROCARE Working Group. Incidence of uveal melanoma in Europe. Ophthalmology. 2007, 114 (12), 2309-2315.
3. Singh, A.D.; Topham, A. Incidence of uveal melanoma in the United States: 1973–1997. Ophthalmology. 2003, 110(5), 956-961.
4. Singh, A.D.; Turell, M.E.; Topham, A.K. Uveal melanoma: trends in incidence, treatment, and survival. Ophthalmology 2011, 118(9), 1881-1885.
5. Houtzagers, L.E.; Ruys, A.; Wierenga, A.P.A.; et al. The relation between iris colour and the development of uveal melanoma. Int J Molecular Sciences 2020, 21(19), 7172.
6. Weis, E.; Shah, C.P.; Lajous, M.; Shields, J.A.; Shields, C.L. The association between host susceptibility factorsand uveal melanoma: A meta-analysis.Arch. Ophthalmol. 2006,124, 54–60.
7. Damato, E.M.; Damato, B.E. Detection and time to treatment of uveal melanoma in the United Kingdom: an evaluation of 2384 patients. Ophthalmology. 2012, 119(8), 1582-1589.
8. Dogrusöz, M.; Jager, M.J.; Damato, B. Uveal melanoma treatment and prognostication. Asia Pac. J. Ophthalmol.2017,6, 186–196.
9. Jager, M.J.; Shields, C.L.; Cebulla, C.M.; et al. Uveal melanoma. Nat. Rev. Dis. Prim. 2020, 6(1), 1-25.
10. Augsburger, J.J.; Corrêa, Z.M.; Shaikh, A.H. Quality of evidence about effectiveness of treatments for metastatic uveal melanoma. Trans Am Ophthalmol Soc. 2008, 106, 128-137.
11. Roelofsen, C.D.M.; Wierenga, A.P.A.; van Duinen, S.; et al. Five Decades of Enucleations for Uveal Melanoma in One Center: More Tumors with High Risk Factors, No Improvement in Survival over Time. Ocul Oncol Pathol. 2021, 7(2), 133-141.
12. McLaughlin, C.C.; Wu, X.C.; Jemal, A.; et al. Incidence of noncutaneous melanomas in the US. Cancer: Interdisciplinary International Journal of the American Cancer Society. 2005, 103(5), 1000-1007.
13. Shields, C.L.; Kaliki, S.; Furuta, M.; et al. Clinical spectrum and prognosis of uveal melanoma based on age at presentation in 8,033 cases. Retina 2012, 32, 1363–1372.
14. Miyamoto, C.; Balazsi, M.; Bakalian, S.; et al. Uveal melanoma: Ocular and systemic disease. Saud. J. Ophthalm. 2012, 26(2), 145-149.

15. Callender, G. R. Malignant melanotic tumors of the eye: a study of histologic types in 111 cases. Trans. Am. Acad. 1931, Ophthalmol. Otolaryngol. 36, 131–140.
16. Kujala, E.; Mäkitie, T.; Kivelä, T. Very long-term prognosis of patients with malignant uveal melanoma. Invest Ophthalmol Vis Sci 2003, 44, 4651–4659.
17. Shields, C.L.; Furuta, M.; Thangappan, A.; et al. Metastasis of uveal melanoma millimeter-by-millimeter in 8033 consecutive eyes. Arch Ophthalmol 2009, 127,989–998.
18. Edge, S.B.; Compton, C.C. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Annals of surgical oncology. 2010, 17(6), 1471-1474.
19. Kivelä, T.; Kujala, E. Prognostication in eye cancer: the latest tumor, node, metastasis classification and beyond. Eye. 2013, 27(2), 243-252.
20. Zloto, O.; Pe'er, J.; Frenkel, S. Gender differences in clinical presentation and prognosis of uveal melanoma. Invest Ophthalmol Vis Sci 2013, 54, 652–656.
21. Prescher, G.; Bornfeld, N.; Horsthemke, B.; et al. Chromosomal aberrations defining uveal melanoma of poor prognosis. Lancet. 1992, 339, 691–692.
22. White, V. A.; Chambers, J. D.; Courtright, P. D.; et al. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. Cancer. 1998, 83, 354-359.
23. Kilic, E.; Naus, N.C.; van Gils, W.; et al. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. Invest Ophthalmol Vis Sci. 2005, 46(7), 2253-2257.
24. Damato, B.; Coupland, S.E. Translating uveal melanoma cytogenetics into clinical care.Arch. Ophthalmol 2009,127, 423–429.
25. Dogrusöz, M.; Jager, M.J. Genetic prognostication in uveal melanoma. Acta Ophthalmol 2018, 96, 331–347.
26. Shields, C.L.; Say, E.A.; Hasanreisoglu, M.; et al. Cytogenetic abnormalities in uveal melanoma basedon tumor features and size in 1059 patients: The 2016 W. Richard Green Lecture.Ophthalmology. 2017,124, 609–618.
27. Kilic, E.; van Gils, W.; Lodder, E.; et al. Clinical and cytogenetic analyses in uveal melanoma. Investig. Ophthalmol. Vis. Sci 2006, 47, 3703–3707.
28. Scholes, A.G.; Damato, B.E.; Nunn, J.; et al. Monosomy 3 in uveal melanoma: correlation with clinical and histologic predictors of survival. Invest Ophthalmol Vis Sci. 2003, 44, 1008-1011.

29. De Lange, M.J.; van Pelt, S.I.; Versluis, M.; et al. Heterogeneity revealed by integrated genomic analysis uncovers a molecular switch in malignant uveal melanoma. Oncotarget. 2015, 6(35), 37824-37835.
30. Versluis, M.; de Lange, M.J.; van Pelt, S.I., et al. Digital PCR validates 8q dosage as prognostic tool in uveal melanoma. PLoS One. 2015, 10(3):e0116371.
31. Sisley, K.; Rennie, I.G.; Parsons, M.A.; et al. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. Genes Chromosomes Cancer. 1997, 19(1), 22-8.
32. Cassoux, N.; Rodrigues, M.J.; Plancher, C.; et al. Genome-wide profiling is a clinically relevant and affordable prognostic test in posterior uveal melanoma. Br. J. Ophthal. 2014, 98(6), 769-774.
33. Gezgin, G.; Dogrusöz. M.; van Essen, T.H.; et al. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Can. Immunol. Immunother. 2017, 66(7), 903-912.
34. Vader, M.J.C.; Madigan, M.C.; Versluis, M.; et al. GNAQ and GNA11 mutations and downstream YAP activation in choroidal nevi. Br. J. Cancer. 2017, 117(6), 884-887.
35. Van Raamsdonk, C.D.; Bezrookove, V.; Green Get Bauer, J.; et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature 2009, 457, 599–602.
36. Van Raamsdonk, C.D.; Griewank, K.G.; Crosby, M.B.; et al. Mutations in GNA11 in uveal melanoma. NEJM. 2010, 363(23), 2191-2199.
37. De Lange, M.J.; Razzaq, L.; Versluis, M.; et al. Distribution of GNAQ and GNA11 mutation signatures in uveal melanoma points to a light dependent mutation mechanism. PloS one. 2015, 10(9), e0138002.
38. Piaggio, F.; Tozzo, V.; Bernardi, C.; et al. Secondary Somatic Mutations in G-Protein-Related Pathways and Mutation Signatures in Uveal Melanoma. Cancers (Basel). 2019, 11(11), 1688.
39. O'Hayre, M.; Degese, M.S.; Gutkind, J.S. Novel insights into G protein and G protein-coupled receptor signaling in cancer. Curr Opin Cell Biol. 2014, 27, 126-35. doi: 10.1016/j.ceb.2014.01.005.
40. Jensen, D.E.; Proctor, M.; Marquis, S.T.; et al. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression.Oncogene. 1998 Mar 5; 16(9):1097-112.
41. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; et al. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science. 2010, 330(6009), 1410-1413.

42. Van Essen, T.H.; van Pelt, S.I.; Versluis, M.; et al. Prognostic parameters in Uveal Melanoma and their association with BAP1 expression. Br J Ophthalmol, 2014, 98 (12), 1738-1743.
43. Koopmans, A.E.; Verdijk, R.M.; Brouwer, R.W.; et al. Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. Modern pathology. 2014, 27(10), 1321-1330.
44. Robertson, A.G.; Shih, J.; Yau, C.; et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32, 204-220
45. Stein-Streilein, J; Taylor, A.W. An eye's view of T regulatory cells. J. Leukoc. Biol 2007 81, 593-598.
46. Apte, R.S.; Sinha, D.; Mayhew, E.; et al. Cutting edge: role of macrophage migration inhibitory factor in inhibiting NK cell activity and preserving immune privilege. J. Immunol 1998 160(12).5693-5696.
47. Yamada, Y.; Sugita, S.; Horie S.; et al. Mechanisms of immune suppression for CD8+ T cells by human corneal endothelial cells via membrane-bound TGFβ. IOVS 2010, 51(5). 2548–2557.
48. Sugita, S. Role of ocular pigment epithelial cells in immune privilege. Archivum immunologiae et therapiae experimentalis. 2009, 57(4), 263-268.
49. Sugita S., Streilein J. W. Iris pigment epithelium expressing CD86 (B7-2) directly suppresses T cell activation in vitro via binding to cytotoxic T lymphocyte-associated antigen 4. Journal of Experimental Medicine. 2003, 198(1):161–171.
50. Sugita S., Futagami Y., Smith S. B., Naggar H., Mochizuki M. Retinal and ciliary body pigment epithelium suppress activation of T lymphocytes via transforming growth factor beta. Exp. Eye Res 2006, 83(6), 1459–1471.
51. Streilein, J.W. Ocular immune privilege and the Faustian dilemma. The Proctor lecture. Investigative ophthalmology & visual science. 1996, 37(10), 1940-1950.
52. Colotta, F.; Allavena, P.; Sica, A.; et al. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis. 2009, 30(7), 1073-1081.
53. Mlecnik, B.; Bindea, G.; Pagès, F.; et al. Tumor immunosurveillance in human cancers. Cancer Metastasis Rev. 2011, 30(1), 5-12.
54. De Cruz, J.r. P.O.; Specht, C.S.; McLean, I.W. Lymphocytic infiltration in uveal malignant melanoma. Cancer. 1990, 65(1), 112-115.
55. Mäkitie, T.; Summanen, P.; Tarkkanen, A.; et al. Tumor-infiltrating macrophages (CD68+ cells) and prognosis in malignant uveal melanoma. Investigative ophthalmology & visual science. 2001, 42(7), 1414-1421.

56. Meecham, W.J.; Char, D.H.; Kaleta-Michaels, S. Infiltrating lymphocytes and antigen expression in uveal melanoma. Ophthalmol. Res. 1992, 24, 20–26.
57. Tobal, K.; Deuble, K.; McCartney, A; et al .Characterization of cellular infiltration in choroidal melanoma. Melanoma Res. 3, 63–65 (1993).
58. De Waard-Siebinga, I.; Hilders, C.G.; Hansen, B.E.; et al. HLA expression and tumor-infiltrating immune cells in uveal melanoma. Graefe's Arch. Clin. Exp. Ophthalmol. 1996, 234, 34–42.
59. Maat, W.; Ly, L.V.; Jordanova, E.S.; et al. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Invest Ophthalmol Vis Sci. 2008, 49(2), 505-10.
60. De Waard-Siebinga, I.; Houbiers, J.G.; Hilders, C.G.; et al. Differential expression of HLA-A and B-alleles on uveal melanoma as determined by immuno-histology. Ocul Immunol Inflamm. 1996, 4(1), 1-14.
61. Jager, M.J.; Hurks, H.M.; Levitskaya, J.; et al. HLA expression in uveal melanoma: there is no rule without some exception. Human immunology. 2002, 63(6), 444-451.
62. Bronkhorst, I.H.; Ly, L.V.; Jordanova, E.S.;et al.. Detection of M2-macrophages in uveal melanoma and relation with survival. Investigative ophthalmology & visual science. 2011, 52(2), 643-650.
63. Ksander, B.R.; Rubsamen, P.E.; Olsen, K.R.; et al. Studies of tumor-infiltrating lymphocytes from a human choroidal melanoma. Invest. Ophthalmo. Vis. Sci. 1991 32(13), 3198-3208.
64. Apte, R. S.; Niederkorn, J. Y. Isolation and characterization of a unique natural killer cell inhibitory factor present in the anterior chamber of the eye. J. Immunol 1996, 156, 2667–2673.
65. Ma, D.; Niederkorn, J.Y. Transforming growth factor-β down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytolysis. Immunology. 1995, 86, 263–269.
66. Niederkorn, J.Y. Ocular immune privilege and ocular melanoma: parallel universes or immunological plagiarism?. Frontiers in immunology. 2012, 13; 3, 148.
67. Mills, C.D.; Kincaid, K.; Alt, J.M.; et al. M-1/M-2 macrophages and the Th1/Th2 paradigm. The Journal of immunology. 2000, 164(12), 6166-6173.
68. Mantovani, A.; Sozzani, S.; Locati, M.; et al. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends in immunology. 2002, 23(11), 549-555.
69. Nesbit, M.; Schaider, H.; Miller, T.H.; et al. Low-level monocyte chemoattractant protein-1 stimulation of monocytes leads to tumor formation in nontumorigenic melanoma cells. J. Immunol. 2001, 166, 6483–6490.

70. Azenshtein, E.; Luboshits, G.; Shina, S.; et al. The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. Cancer Res.2002, 62, 1093–1102
71. Bronkhorst, I.H.; Vu, T.K.; Jordanova, E.S.; et al. Different subsets of tumor-infiltrating lymphocytes correlate with macrophage influx and monosomy 3 in uveal melanoma. Invest. Ophthal. Vis. Sci. 2012, 53(9), 5370-5378.
72. De Lange, M.J.; Nell, R.J.; Lalai, R.N.; et al. Digital PCR-Based T-cell Quantification–Assisted Deconvolution of the Microenvironment Reveals that Activated Macrophages Drive Tumor Inflammation in Uveal Melanoma. Mol. Canc. Res. 2018, 16(12), 1902-1911.
73. Biswas, S.K.; Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010, 11(10), 889-896.
74. Yu, P.; Fu, Y.X. Tumor-infiltrating T lymphocytes: friends or foes? Lab Invest. 2006, 86, 231–245.
75. Mihm, M.C.; Mulé, J.J. Reflections on the histopathology of tumor-infiltrating lymphocytes in melanoma and the host immune response. Cancer Immunol Res. 2015, 3, 827–835.
76. De la Cruz, P.O.J.r.; Specht, C.S.; et al. Lymphocytic infiltration in uveal malignant melanoma. Cancer. 1990, 65(1), 112-115.
77. Whelchel, J.C.; Farah, S.E.; McLean, I.W.; et al. Immunohistochemistry of infiltrating lymphocytes in uveal malignant melanoma. Invest Ophthalmol Vis Sci. 1993, 34(8), 2603-2606.
78. Lagouros, E.; Salomao, D.; Thorland, E.; et al. Infiltrative T regulatory cells in enucleated UMs. Trans Am Ophthalmol Soc. 2009, 107, 223–228.
79. Goldsby, R.A.; Kindt, T.J.; Osborne, B.A.; et al. Immunology. 5th ed. Mc Graw Hill; 2003, 171–194.
80. Szeto, C.; Lobos, C.A.; Nguyen, A.T.; et al. TCR Recognition of Peptide–MHC-I: Rule Makers and Breakers. Int. J. Mol. Scie. 2021, 22(1), 68-94.
81. Roche, P.A.; Furuta, K. The ins and outs of MHC class II-mediated antigen processing and presentation. Nature Reviews Immunology. 2015, 15(4), 203-216.
82. Garrido, F.; Cabrera, T.; Concha, A.; et al. Natural history of HLA expression during tumour development. Immunol Today. 1993, 14(10), 491-499.
83. Blom, D.J.; Luyten, G.P.; Mooy, C.; et al. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Invest. Ophthalmo. Visual sci. 1997, 38(9), 1865-1872.
84. Ericsson, C.; Seregard, S.; Bartolazzi, A.; et al. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Invest Ophthalmol Vis Sci. 2001, 42(10), 2153-2156.

85. Hurks, H.M.H.; Metzelaar-Blok, J.A.W.; Mulder, A. et al. High frequency of allele-specific down-regulation of HLA Class I expression in uveal melanoma cell lines. Int. J. Cancer 2000, 85, 697-702.
86. Anastassiou, G.; Rebmann, V.; Wagner, S.; et al. Expression of classic and nonclassic HLA class I antigens in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2003, 44, 2016–2019.
87. De Waard-Siebinga, I.; Creyghton, W.M.; Kool, J.; et al. Effects of interferon alfa and gamma on human uveal melanoma cells in vitro. Br. J. Ophthalmol. 1995, 79, 847–855.
88. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009, 1(6), a001651.
89. Oh, H.; Ghosh, S. NF-κB: roles and regulation in different CD4 (+) T-cell subsets. Immunol Rev. 2013, 252(1), 41-51.
90. Pires, B.R.B.; Silva, R.C.M.C.; Ferreira, G.M.;. NF-kappaB: Two Sides of the Same Coin. Genes (Basel). 2018, 9(1), 24.
91. Meir, T.; Dror, R.; Yu, X.; et al. Molecular characteristics of liver metastases from uveal melanoma. Invest Ophthalmol Vis Sci. 2007, 48(11), 4890-4896.
92. Dror, R.; Lederman, M.; Umezawa, K.; et al. Characterizing the involvement of the nuclear factor-kappa B (NF kappa B) transcription factor in uveal melanoma. Invest Ophthalmol Vis Sci. 2010, 51(4), 1811-1816.
93. Halkidou, K.; Gaughan, L.; Cook, S. et al. Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate. 2004, 59, 177–189.
94. Song, J.; Noh, J.H.; Lee, J.H.; et al. Increased expression of histone deacetylase 2 is found in human gastric cancer. APMIS. 2005;113:264–268
95. Wahid F, Shehzad A, Khan T, Kim YY. MicroRNAs: synthesis, mechanism, function, and recent clinical trials. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2010; 1803(11):1231-43.
96. Kim, J.; Ya, F.; Xiao, Z.; et al. MicroRNAs and metastasis: small RNAs play big roles. Cancer. Met. Rev. 2018, 37(1), 5-15.
97. Volinia, S.; Calin, G.A.; Liu, C.G.; et al. A microRNA expression signature of human solid tumors defines cancer gene targets. PNAS. 2006, 103(7), 2257-2261.
98. Wu, F.; Zikusoka, M.; Trindade, A.; et al. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2α. Gastroenterology. 2008, 135(5), 1624-1635.

99. Worley, L.A.; Long, M.D.; Onken, M.D.; Harbour, J.W. Micro-RNAs associated with metastasis in uvealmelanoma identified by multiplexed microarray profiling.Melanoma Res.2008,18, 184–190.

100. Smit, K.N.; Chang, J.; Derks, K.; Vaarwater, J.; Brands, T.; Verdijk, R.M.; Wiemer, E.A.C.; Mensink, H.W.;Pothof, J.; de Klein, A.; et al. Aberrant MicroRNA Expression and Its Implications for Uveal Melanoma Metastasis. Cancers 2019,11, 815.

101. Aughton, K.; Kalirai, H.; Coupland, S.E. MicroRNAs and Uveal Melanoma: Understanding the Diverse Role of These Small Molecular Regulators. Int. J. Mol. Sci. 2020, 21(16), 5648.

102. Alegre, M. L.; Frauwirth, K. A.;Thompson, C. B., T-cell regulation by CD28 and CTLA-4. Nat. Rev. Immunol. 2001. 1, 220–228.

103. Topalian, S. L.; Drake, C. G.; Pardoll, D. M. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr. Opin. Immunol. 2012. 24, 207–212

104. Rodrigues, M.; Koning, L.; Coupland, S.E.; et al. UM Cure 2020 Consortium. So Close, yet so Far: Discrepancies between Uveal and Other Melanomas. A Position Paper from UM Cure 2020. Cancers (Basel). 2019, 11(7), 1032.

105. Durante, M.A.; Rodriguez, D.A.; Kurtenbach, S.; et al. Single-cell analysis reveals new evolutionary complexity in uveal melanoma. Nat. Commun. 2020, 11(1), 1-10.

106. Triebel, F.; Jitsukawa, S.; Baixeras, E.; et al. "LAG-3, a novel lymphocyte activation gene closely related to CD4." TJ Exp Med. 1990, 171(5), 1393-1405

107. Krishnakumar, S.; Abhyankar, D.; Lakshmi, S.A.; et al. HLA class II antigen expression in uveal melanoma: Correlation with clinicopathological features. Exp. Eye Res. 2003, 77:175–180.

108. Jager, M.J.; van der Pol, J.P.; de Wolff-Rouendaal, D.; et al. Decreased expression of HLA Class II antigens on human uveal melanoma cells after in vivo X-ray irradiation. Am. J. Ophthalmol. 1988, 105:78–8