Pigmentation and prognosis in uveal melanoma

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Chapter 1: INTRODUCTION AND OUTLINE

Uveal Melanoma: Current Evidence on Prognosis, Treatment and Potential Developments

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ABSTRACT

Uveal Melanoma (UM) is a rare disease, yet it is the most common primary intraocular malignancy in adult patients. Despite continuous advancements and research, the risk of metastasis remains high. It is possible to stratify patients according to their risk of metastases using a variety of known risk factors. Even though there is no gold standard for the prognostication of patients with UM, it is becoming increasingly clear that combining histo-pathological, patient-related and molecular prognostic markers allows a more accurate prediction of the metastatic risk than by using only one of these parameters. Primary UM in the eye can be treated effectively with eye-sparing radiation-based techniques or enucleation. However, it is not yet possible to prevent or treat metastases with the current therapeutic options. Nonetheless, the efforts to find new therapeutic targets continue and progress is being made, especially in the field of targeted therapy, as exemplified by the anti-gp100 bispecific molecule Tebentafusp. We here look into the history of UM, its incidence, presentation and diagnosis, the known prognostic factors and the treatment options, both for the primary tumour and for metastases. Different populations may have different risks for developing UM, and that each country should evaluate their own patients.

INTRODUCTION

Uveal Melanoma (UM) is a malignant tumour that arises in the uvea. The word "melanoma" comes from the Greek word for black (μελας) and the suffix -ωμα, which indicates swelling, and was first introduced by Carswell in 1838 to describe any type of black discolouration.[1] The word "uvea" comes from the Medieval Latin, where the word "uvea" was used to describe something "resembling a grape" ("uva" in Latin). The terminology is sometimes confusing, as the terms uveal and choroidal melanoma are often used without a clear definition and earlier sources use the word "sarcoma" to describe lesions that today we would call uveal melanomas.[2-5] However, uveal melanoma nowadays stands for any malignant lesion derived from melanocytes in the uveal tissues (the iris, the ciliary body and the choroid), and the term "choroidal melanoma" should be reserved for lesions originating in the back of the eye. In order to prevent misunderstanding, in the following text we will apply the term uveal melanoma also to lesions that match this definition but were previously described under different names.

The earliest record of a case suggestive of a UM dates back to the 16th century[6], when the German surgeon Georg Bartisch described a fungating lesion growing out of the eye of a patient and described instruments to remove the eye.[7] The first detailed accounts came in the early 19th century[8], when two Scottish surgeons, James Wardrop and Allan Burns, examined and followed an adult patient with a dark brown intraocular tumour and liver involvement and wrote separate reports, which described the lesion and the clinical history in detail.[9, 10] The 19th century brought along further developments in the field of ocular oncology: the invention of the ophthalmoscope by Helmholtz in 1850 and the first considerations on the prognosis of UM, though with slightly different positions. In 1868, Albrecht von Graefe described a case series of 150 eye tumours and stated that the prognosis of UM was entirely unfavourable and that enucleation did not benefit survival.[2, 3] In the same year, however, Hermann Knapp published a textbook on intraocular tumours, in which he reported 15 cases of eye melanoma and stated that "the operation undertaken in the first stages of the formation of the tumour will with certainty remove and cure the affection".[4] Later works by Ernst Fuchs further corroborated the grim prognosis of UM and advised enucleation as early as possible, if the tumour was confined to the eyeball.[5] George Callender was the first to study the cytologic features of UM and to correlate them to survival.[11] He was also the first to report the presence of spindle and epithelioid cells inside the tumours.

Incidence

UM is a rare disease, and yet it is the most common primary intraocular malignancy in adults. Its incidence varies across the globe and has been fairly stable over time: it has the highest incidence in Northern Europe, Western Europe and Oceania (> 8 cases per million person-years), an intermediate one in North America, Eastern Europe and Southern Europe (2-7.9 cases per million person-years) and is rare in South America, Asia and Africa (< 2 cases per million person-years).[12-14] Even within Europe, there is a great variation in the incidence of UM, with a decreasing gradient from North to South, with The Netherlands being among the countries with the highest incidence.[12, 14] This geographical distribution points to a different predisposition in different populations, and in particular, to a higher risk in people with pale skin and light eyes.[12, 14] Indeed, the presence of blue or green eyes has repeatedly been reported as a risk factor for the development of UM.[15, 16] To further corroborate the link between eye colour and UM development, two single nucleotide polymorphisms (SNPs) that are associated with eye colour have been associated with UM risk (rs12913832 in *HERC2* and rs12203592 in *IRF4*).[17, 18] One may also postulate that Ultraviolet (UV) light, which is known to increase the risk of

cutaneous melanoma, may have a role in the pathogenesis of UM as well. However, the lack of convincing evidence and the lack of a genetic UV signature in UM cells discredits this hypothesis.[19-22]

Presentation and diagnosis

UM most frequently arises in the choroid (90%) and less frequently in the ciliary body (6%) and iris (4%).[23] At presentation, about 30% of the patients are asymptomatic, but most people seek medical attention because of symptoms such as blurred or distorted vision, visual field defects or photopsia.[24, 25] Patients with ciliary body UM or large, anteriorly-placed choroidal melanomas may report pain, and patients with iris melanomas may notice a visible mass or a change in iris colour.[25] The anterior location of the iris and the possibility to notice alterations with the naked eye, especially in people with light eyes, leads to an earlier reporting, diagnosis and treatment of melanomas that arise in this location. It may be that because of the early diagnosis, iris tumours are usually not malignant at diagnosis.[26]

Clinical examination is performed primarily with slit lamp biomicroscopy and indirect ophthalmoscopy, aided by imaging techniques such as fundus photography, fundus autofluorescence, ultrasonography, optical coherence tomography (OCT), Magnetic Resonance Imaging (MRI) and fluoresceine or indocyanine green angiography. Ultrasonography reveals the size, shape and internal structure of the tumour, and it can also reveal other features such as a "mushroom" configuration, which suggests a rupture in Bruch's membrane. OCT scans can be used to visualize posterior ocular structures in detail, such as the retina and choroid, and is very useful in diagnosing subretinal fluid. For larger tumours, MRI is a better option. Fluoresceine angiography can be used to assess vascular structures and leakage patterns.

When one cannot decide whether a lesion is a small malignant melanoma or a benign choroidal naevus, ophthalmologists can rely on a set of risk factors related to malignant transformation of choroidal naevi, such as tumour size, documented growth, the presence of subretinal fluid, the presence of orange pigment and a low internal reflectivity on ultrasound. Several acronyms exist to help classify uncertain lesions, such as TFSOM-DIM (To-find-small-ocular-melanomas-doing-imaging: Thickness greater than 2 mm, Fluid under the retina, Symptoms, Orange pigment, Melanoma hollow on ultrasonography, and Diameter greater than 5 mm)[27-29] and MOLES (Mushroom shape, Orange pigment, Large size, Enlargement, and Subretinal fluid).[30] In case of challenging differential diagnoses or orbital invasion, CT or MRI can be used, and in case of further doubts, a biopsy of the lesion can be taken.[31, 32]

PROGNOSIS

UM has been reported to have a particularly grim prognosis ever since the first case series were published in the 19th century. While some authors thought that removal of the tumour and the eye would prevent the spread of metastases and death[4], Albrecht von Graefe stated that enucleation did not benefit survival.[2, 3] About 150 years later, we can quite confidently side with von Graefe's view: there have been many improvements and advancements in both diagnosis and treatment of UM, but the prognosis has not improved and there is no convincing evidence that local treatment prevents the spread of metastases once the diagnosis of a UM has been made.[33, 34]

When considering tumours of all sizes, 5-year overall survival rates vary between 76% and 82% [13, 35, 36] while 5-year metastasis-related survival varies between 84% and 91%[35, 36], without relevant differences between cases treated with irradiation or enucleation.[36] However, studies that excluded small UM (thereby including only UM with a thickness of at least 2 mm and diameter of at least 16

mm or any diameter with a thickness of at least 10 mm)[37] or that focused on enucleated cases only, showed worse numbers, with 5-year survival rates around 70%.[34] A study that analysed very long term survival reported a 15-year overall survival rate of 35% and a 15-year UM metastasis-related survival of 55%.[38] Moreover, an analysis performed in a cohort of 1212 patients who were enucleated for a UM in our centre showed no change in survival over the last 50 years.[34] These data suggest that the probability of metastatic spread is established early on in the tumour, even before diagnosis, and we do not currently have any effective method to prevent it. It may be different when considering suspicious pigmented choroidal lesions: early treatment may prevent the development of a melanoma, as postulated by Damato et al.[39] Even though we cannot prevent the development of metastases at the moment, it is possible to predict which patients are more likely to develop metastases through a variety and combination of prognostic factors, some of which are patient-related and some tumour-related. Age is the most relevant patient-related factor, not only in overall survival, but also in UM-related survival: patients with an older age at diagnosis have a shorter survival than younger patients.[40-43]

Tumour features

Tumour-related features can be divided in histo-pathological and molecular characteristics.

Histo-pathological features

A larger tumour diameter and greater tumour thickness have repeatedly been associated with a higher risk of metastases and a shorter survival[23, 35, 36, 40, 43-45], as have been the presence of ciliary body involvement and extra-scleral extension, which are manifestations of local invasiveness.[35, 38, 42, 43, 46, 47] These tumour-related features have been shown to be more informative when used in combination. The Collaborative Ocular Melanoma Study Group has defined criteria based on largest basal diameter and thickness to classify tumours as small, medium or large.[48, 49] The Tumour-Node-Metastases (TNM) / American Joint Committee on Cancer (AJCC) staging uses tumour diameter and thickness to define four T size categories (T1 to T4) and subsequently adds information on ciliary body involvement and extraocular extension to define stages of increasing risk for death by UM metastases (T1a to T4e).[50] The presence of extraocular extension larger than 5 mm automatically classifies a tumour as T4e. Next, the presence of lymph node and distant metastases is factored in and the T stages are grouped in AJCC stages with progressively worse prognosis (I to IV). Stage IV is reserved for patients with metastases to lymph nodes or distant sites at diagnosis, independent of the T stage.

Another tumour-related feature associated with a high mortality is the presence of epithelioid cells compared to spindle cells.[43, 44, 47] Callender was the first to report the prognostic implications of specific cytologic features of UM and he designed a UM classification based on cell type, a simplified version of which is still being used today.[11] While the World Health Organisation recognises five cell types[51], pathologists usually classify UM as one of three categories: epithelioid, spindle or mixed (if it contains at least 10% of cells of either cell type). A high number of mitotic figures in the tumour, which marks the proliferative activity of tumour cells, has also been associated with a worse prognosis.[42, 47]

Histology can also be used to study blood vessels inside the tumour. Microvascular density can be studied by staining for blood vessel markers such as CD31, CD34 and von Willebrand factor, and is predictive of a worse prognosis.[52, 53] In addition, UM can have extravascular loops and networks, which are better visualised with periodic acid-Schiff stain and a dark green filter and carry a poor prognosis.[54, 55]

In terms of tumour cell invasion, ingrowth in blood vessels is associated with a high rate of metastasis and a worse survival, but it is also associated with other prognostic factors such as a larger tumour size and the presence of loops and networks.[56, 57]

Chromosome status

Other, more recent prognostic factors come from molecular analysis of tumour tissue or tumour cells. Chromosomal aberrations associated with a poor prognosis are the following: monosomy of chromosome 3 (M3) compared to disomy 3 (D3), the presence of extra copies of the long arm of chromosome 8 (8q gain) compared to the normal two copies, loss of the short arm of chromosome 1 (1p).[58-63] Conversely, patients with tumours with gain of the short arm of chromosome 6 (6p gain) have a better prognosis than those with two copies of chromosome 6p.[59, 62]

As already noticed early on by White and Damato, combining chromosome alterations gives a better prognostication than focusing on single copy number variations.[59, 62] The Cancer Genome Atlas (TCGA) study analysed chromosome copy number, mutational status, mRNA expression and methylation patterns in 80 UM samples from different centres worldwide.[64] Using chromosome 3 status as well as chromosome 8q status has led to the identification of four genetic subtypes with different survival and tumour features that were later named groups A-B-C-D (Table 1a).[65] Group A includes UM with D3 and normal 8q copy number, group B includes UM with D3 and 8q gain, group C includes UM with M3 and 8q gain (1 extra copy) and group D includes UM with M3 and 8q amplification (2 or more extra copies). In addition to confirming the benefit of combining chromosome information, the TCGA study also remarked that there is a difference in survival and tumour features between tumours with different numbers of extra 8q copies.[66]

Mutations and RNA

Many types of cancer harbour many mutations, but UM is known to have a low tumour mutational burden.[21] The primary driver mutation of UM, which is also present in choroidal naevi, is a mutation activating the Galpha-q pathway, which may occur in G Protein Subunit Alpha Q (*GNAQ*), G Protein Subunit Alpha 11 (*GNA11*), Cysteinyl Leukotriene Receptor 2 (*CYSLTR2*) or Phospholipase C Beta 4 (*PLCB4*).[20, 22, 67-70] These mutations are usually mutually exclusive and are generally considered not to have an impact on prognosis.

A few mutually-exclusive genetic mutations have been linked to prognosis in UM (Table 1a). Inactivating mutations in BRCA1 Associated Protein 1 (*BAP1*) (located at chromosome 3p21.1) occur throughout the entire gene and are strongly associated with monosomy of chromosome 3, a poor prognosis and early development of metastases. Mutations in the Splicing Factor 3b Subunit 1 (*SF3B1*) (located at 2q33.1) usually affect codon 625, are associated with a better prognosis compared to *BAP1* mutations, and are associated with gain of 8q and with the development of late metastases.[21, 71, 72] The most prognostically-favourable mutations are in exon 1 and 2 of Eukaryotic Translation Initiation Factor 1A X-Linked (*EIF1AX*) (located on Xp22), which are usually not associated with any copy number alteration.[72-74]

A further method that is being used for prognostication of UM is gene expression profiling (GEP), which uses the mRNA expression of a selected panel of 15 genes to assign patients to one of two classes (Table 1b).[75-80] Tumours in GEP class 1 have a lower risk of metastases and better survival compared to tumours in GEP class 2, which frequently have M3, a *BAP1* mutation and several other negative prognostic factors. Each of these two classes can be further sub-divided into two sub-classes with different characteristics.[66, 81, 82] The expression of cadherin 1 (*CDH1*) and RAS oncogene family

(*RAB31*) distinguishes class 1A and class 1B, while inflammatory markers and chromosome 8p copy number differ between class 2A and class 2B.

A gene that has been of interest in the past decade is *PRAME* (Preferentially Expressed Antigen in Melanoma). *PRAME* is a cancer-testis antigen, and its expression is increased and associated to a worse prognosis in several types of cancer, among which is UM.[83] Several groups have shown that *PRAME* expression is independently correlated to a shorter survival, both when considering all UM cases in a cohort and within GEP class 1 and class 2 separately. Moreover, class 2 UM have a higher *PRAME* expression than class 1B UM, which in turn have a higher *PRAME* expression than class 1A UM.[84-87] Further evidence of the continuing interest in this gene, the Collaborative Ocular Oncology Group 2 (COOG2) study, will include GEP class, mutations in *BAP1*, *SF3B1*, and *EIF1AX* and expression of *PRAME*, aiming at providing a useful genetic classification system.[88] PRAME is not only of prognostic significance, but can also be a target for immunotherapy.[86]

As expected, molecular prognostic factors frequently overlap with each other and with histo-pathological features. GEP class 1 UM usually have D3 and lack a *BAP1* mutation, whereas GEP class 2 UM frequently have M3 and a *BAP1* mutation.[75, 77] Moreover, prognostically bad histo-pathological prognostic features such as a larger tumour size, epithelioid cell type, extravascular loops, and a higher proliferation rate, are more frequent in UM with M3/ BAP1 loss/ GEP class 2, compared to their more benign counterparts.[61, 62, 71, 81] Most of these studies are based on cohorts of mainly patients of white European origin, as they are more prone to developing UM.

Combining prognostic markers

The possibility to combine prognostic systems to improve prognostication has been explored by several authors, with positive results. Bagger, Dogrusöz as well as Negretti showed that combining information from the AJCC size classification with chromosome status achieved a greater prognostic accuracy than either system independently.[89-91] Most work on prognostication has been performed in centres where many patients are being treated: such centres are especially located in countries where many of the inhabitants have light eyes, with an increased risk of developing UM.

The Liverpool Uveal Melanoma Prognosticator Online (LUMPO) algorithm is an online system that allows personalized prognostication based on a large number of parameters. In its latest iteration, it includes sex, age at treatment, anterior margin position, largest basal diameter, thickness, extraocular spread, cell type, the presence of closed loops, mitotic count and two molecular factors: monosomy of chromosome 3 and gain of 8q.[92, 93] Algorithms such as these need to be assessed for populations without a white Northern European background.

Inflammation

Differently from other tumour types, the presence of an inflammatory infiltrate is associated with poor prognosis in UM. The presence of tumour-infiltrating macrophages and lymphocytes is associated with adverse prognostic factors, such as the presence of epithelioid cells, a higher microvascular density and monosomy of chromosome 3.[94-98] In addition, evidence from previous studies suggests that the UM microenvironment may be immunosuppressive. Our group demonstrated that the immune infiltrate included mostly anti-inflammatory M2 macrophages and several subsets of T cells, including regulatory T cells.[97] Figueiredo et al. reported an upregulation of immunosuppressive genes found in UM with BAP1 loss compared to UM without BAP1 loss, while Durante et al. analysed scRNAseq data and showed the expression of the immune checkpoint lymphocyte-activation gene 3 (LAG3) on tumour-infiltrating CD8+ T cells.[99, 100] Gezgin demonstrated

that tumour-infiltrating CD4+ and CD8+ cells could only respond against UM cells when separated from their environment. We do not yet know whether the downregulation of T cells is due to the presence of UM cells, surrounding fibroblasts, myeloid-derived suppressors cells or any specific cytokine or other suppressive factors.[101]

PIGMENTATION

UM originate from melanocytes in the choroid and can have variable degrees of pigmentation, from non-pigmented to extremely dark.

The degree of tumour pigmentation can be evaluated macroscopically, either after enucleation or during clinical examination, or microscopically, either by grading the intensity of pigmentation in cells or the proportion of pigmented cells in the tumour.[45, 47, 49, 102] The degree of tumour pigmentation has been linked to prognosis by several authors. Before the advent of molecular prognostic factors, McLean, Packard and Seddon reported that a UM with a heavy microscopic pigmentation carried a worse prognosis than a light UM.[43, 44, 47, 49] However, these authors and the COMS group also reported that dark tumour pigmentation was associated with a larger tumour diameter and a higher number of epithelioid cells.[49] Clinical tumour pigmentation evaluated by fundoscopy was investigated in two more recent studies. The Shields group analysed prognostic factors in patients with different racial backgrounds and identified pigmentation among the factors independently associated with a shorter survival, both in the full cohort including all patients and when considering white patients only.[23] However, this analysis included iris tumours as well and did not include molecular factors in the prognostic models. Markiewicz at el. compared amelanotic tumours and tumours with pigmentation and showed that amelanotic tumours have a longer survival, especially in UM with AJCC stage I and II.[103] They also confirmed the association between tumour pigmentation and bad prognostic features such as epithelioid cells, extrascleral extension and loss of BAP1 protein expression.

The association between tumour pigmentation and other tumour features that are relevant for prognosis makes it harder to evaluate the independent role of pigmentation in prognosis and poses the question whether tumour pigmentation actively influences prognosis or is an innocent bystander. To further complicate the picture, some tumours have a mixed pigmentation, with darker and lighter areas, which can be visualised both by direct observation and by magnetic resonance imaging.[104] This clearly points towards heterogeneity within the tumour, with the simultaneous presence of clones with variable metastatic potential, which complicates the prognostication of patients with UM into a specific prognostic category.[105]

Biology of melanocyte pigmentation

The degree of pigmentation in melanocytes is determined by the amount and type of melanin present in melanosomes, which are lysosomal-related organelles that produce and store melanin.[106] Two types of melanin exist: eumelanin, which is brown-black and has photoprotective and anti-oxidant properties, and pheomelanin, which is yellowish-red and is associated with genotoxic stress. The synthesis of these two types of melanin follows two separate routes that share the first step mediated by tyrosinase (TYR): the oxidation of L-tyrosine to L-DOPA and DOPAquinone (DQ). Subsequently, DQ can undergo cyclisation and be converted to eumelanin in a series of steps mediated by dopachrome tautomerase (DCT, also known as tyrosine-related protein-2, TYRP-2) and tyrosine-related protein-1 (TYRP-1). Alternatively, in the presence of cysteine or glutathione, DQ is converted to pheomelanin.[107] The factors involved in melanin synthesis are strictly regulated, and the master regulator is the microphthalmia-associated transcription factor (MITF). Its role in cutaneous and UM has not been fully

elucidated yet. The process of melanin synthesis and pigmentation has been studied extensively and is well understood for the skin, but less is known about it in eyes.

The quantity of eumelanin and the eumelanin-pheomelanin ratio in uveal melanocytes have been shown to be higher in brown irides than in green and blue irides.[108, 109] The amount of eumelanin and pheomelanin has been investigated in UM cell lines and in uveal melanocytes from eyes with different iris colours: UM cell lines had lower eumelanin content and eumelanin-pheomelanin ratios but similar pheomelanin content when compared to normal uveal melanocytes.[106] These findings led the authors to postulate that the lower eumelanin content made melanocytes more susceptible to UV damage or oxidative stress. While we can discard the hypothesis of a role for UV light because of lack of epidemiological evidence and lack of a UV signature in UM cells, we may still consider oxidative damage as a potential factor associated with UM development. This theory, however, does not explain why darker tumours are associated with a worse prognosis.

Iris colour

While we do not know what determines the level of pigmentation of UM, we know that iris colour is genetically determined. The most important locus for eye colour determination is the HERC2-OCA2 locus on chromosome 15, and the single nucleotide polymorphism (SNP) universally recognised as most relevant is rs12913832 (*HERC2*), which mainly distinguishes brown from blue.[110-112] Other SNPs in other genes such as *OCA2, TYR, SLC45A2* and *IRF4*, when used in addition to rs12913832, have been shown to further improve eye colour prediction.[110, 112, 113] As mentioned earlier, SNPs rs12913832 in *HERC2* and rs12203592 in *IRF4* have been associated with the risk of developing UM.[17, 18] Moreover, a recent study of worldwide incidence and risk factors for ocular melanoma showed a very strong correlation and an evident geographic overlap between the incidence of ocular melanoma, the frequency of blue eye colour and the distribution of rs12913832 alleles.[14]

TREATMENT

Even after centuries of study and technological advances, the treatment of UM is still open to improvement, especially when metastases develop. Local tumour control is achieved very effectively with the options available at the moment, but prevention and treatment of metastatic spread is not successful yet.

Treatment of primary uveal melanoma

The primary tumour in the eye is most frequently treated by radiotherapy, local resection or enucleation. Local resection can be performed ab interno (endoresection) or ab externo (exoresection) and can be performed in isolation or in combination with radiotherapy.[114, 115] Radiotherapy is a globe-sparing technique and can be administered in different ways.[46, 116-118] Brachytherapy is performed by suturing a plaque with a radioisotope such as iodine-125, ruthenium-106 or palladium-103 to the sclera and removing it a number of days later. External beam radiation delivers a targeted beam of radiation to the tumour and it includes proton beam therapy, which uses protons and usually requires suturing tantalum clips on the sclera before irradiation, and stereotactic radio surgery with gamma knife or cyber knife. Each of these options has its own benefits and complications and the choice between these eye-sparing techniques depends mainly on the availability at the treatment centre and on tumour and patient features. In centres where different options are available, external beam therapy tends to be used in patients with tumours that are very large or are very close to the optic nerve.

Each of these techniques, although extremely effective in killing tumour cells, can lead to ocular complications, especially in terms of visual function. An emerging treatment for indeterminate lesions and small choroidal melanomas that is being investigated in clinical trials is light-induced therapy with Belzupacap Sarotalocan (Bel-sar) as a photosensitiser, which aims at specifically killing tumour cells while having a better visual prognosis.[119-121] Experimental work showed that pigmented and non-pigmented cell lines were equally sensitive to treatment with Bel-sar.[122]

Enucleation is usually reserved for cases that are unsuitable for radiotherapy, because of large tumour size, severe loss of vision with a painful eye, evidence of extrascleral invasion, severe ocular complications or adverse events after a prior eye-sparing treatment.

Cell lines

Because of the grim prognosis and the lack of effective strategies to prevent or treat metastatic spread, the search for new therapeutic targets in UM is always ongoing. New potential drugs undergo extensive preclinical testing before reaching the stage of clinical trials. The first step is usually *in vitro* testing in cell lines, followed by *in vivo* testing in animal models.

Several established primary and metastatic UM cell lines are available and are being used worldwide in the search of new therapeutic targets. These UM cell lines are rather heterogeneous in terms of cell type, BAP1 protein expression, mutations and chromosome abnormalities[123-127], hence research groups should be careful in the choice of which cell lines to use in their experiments and make sure that the cell lines are truly representative of UM. In the past, some cell lines originally classified and used as UM cell lines were discovered to have been misidentified: some carried a *BRAF* mutation, which is typical of cutaneous melanoma, some showed contamination by a cutaneous melanoma cell line, and some shared identical short tandem repeat profiles (STR) despite being considered to have derived from different patients.[125, 128-131]

This issue is not only present in UM, but it permeates all aspects of cell line research and it undermines the validity of studies performed on misidentified cell lines.[132-135] Moreover, it may lead to waste of time and resources in unnecessary or misguided investigations, that would have been avoided by thorough and periodical testing of the characteristics and identity of cell lines.

Treatment of metastatic uveal melanoma

While treatment of the primary tumour is extremely successful, prevention and treatment of metastatic disease is rather unsatisfactory. Because there is no gold standard or universally-recognised guideline, treatment of UM metastases is varied and depends on many factors, among which are patient performance status, comorbidities, type and number of metastases and availability.[136] Although it is hard to compare different treatment modalities because of heterogeneity in study designs and populations, liver-directed therapies have better outcomes compared to systemic therapies, and they include hepatic artery infusion chemotherapy, radioactive microsphere administration for selective internal radiation therapy, immune-embolization, transarterial chemoembolization, and localized delivery of chemotherapy by isolated hepatic perfusion and percutaneous hepatic perfusion.[137, 138] However, these techniques cannot be performed in patients with a high number of metastases and with a poor performance status. Systemic therapy options usually include chemotherapy with alkylating agents (dacarbazine or temozolomide) or immune checkpoint inhibitors (either as single agent or as a combination of an anti-CTLA4 and an anti-PD1 agent). Both options are far from ideal, because chemotherapeutic agents have limited benefit and immune checkpoint inhibitors have shown a good response only in patients with a high mutational

burden and a germline mutation in the gene coding for methyl pGbinding domain protein 4 (*MBD4*).[139-144]

A promising option for patients with metastatic UM patients who have HLA-A*02:01 is tebentafusp, which has been shown to prolong overall survival while bringing only limited benefit in terms of objective responses and progression-free survival. Tebentafusp is an Imm-TAC (Immune-mobilising monoclonal TCR against cancer), a bispecific molecule consisting of an anti-CD3 single chain antibody fragment and a monoclonal high-affinity T cell receptor (TCR) targeting the tumour antigen gp100.[136, 145-149] The recognition of gp100 is dependent on presentation by HLA-A*02:01, which limits its use to the subgroup of patients that carry this genetic polymorphism. Studies in Leiden, The Netherlands, show that HLA-A2 occurs in 53-55 % of Dutch UM patients, which is a frequency similar to the Dutch general population.[150, 151]

Therapeutic options that are independent of the HLA type are being studied as well. One example is the combination of protein kinase C inhibitor darovasertib and c-MET inhibitor crizotinib, which has shown good results in terms of objective response and survival in phase 1/2 trials.[152, 153]

Novel approaches consist of combinations of liver-directed and systemic therapies, as exemplified by the CHOPIN trial (NCT04283890) that is being performed at the Leiden University Medical Centre.[154] In addition, researchers worldwide are constantly searching for new therapeutic targets that hopefully will bring new drugs to clinical practice.

CONCLUSION

Uveal melanoma is still a focus of study for many groups around the world, because there are still several questions open and there is much room for improvement of the treatment of metastases. Even though there is no gold standard for the prognostication of patients with UM, it is becoming increasingly clear that combining histo-pathological, patient-related and molecular prognostic markers allows more accurate prediction of metastatic risk. Different populations may show a different sensitivity to develop UM and UM metastases. As for the treatment, local tumour control can be achieved effectively but it is not yet possible to prevent or treat metastases with the current therapeutic options. Nonetheless, the efforts to find new therapeutic targets continue and progress is being made, especially in the field of targeted therapy.

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THIS THESIS

We set out to investigate a number of questions that come up when studying UM. As outlined above, prognostication of UM is at the same time straightforward and complex, because several prognostication systems exist, but none of them gives a perfect prediction and they tend to work better when combined. **Chapter 2.1** provides a detailed overview of prognostic factors, while **chapter 2.2** focuses on the benefit of combining the TCGA and the AJCC systems. Our data show that a combination of prognostic systems provides better prognostication of tumours, especially for those in the intermediate categories.

In **chapter 3**, we focus on an extremely intriguing and puzzling feature of eyes and UM: pigmentation. We first tackle eye colour, and we set out to check if iris colour, which is one of the main risk factors for UM development, also bears prognostic significance. Next, we investigate the association between eye colour and tumour pigmentation. We first use clinically-determined and self-reported eye colour (**chapter 3.1**), and subsequently use a set of six eye colour-related single nucleotide polymorphisms (SNPs) (**chapter 3.2**) and identify one SNP in *HERC2* as related to prognosis.

Prior studies on tumour pigmentation suggest that dark tumours give a poor prognosis, but that they also contain more epithelioid cells and are larger than light tumours. Therefore, the question is still open: does melanin have an active role in the metastatic process or is it an innocent bystander? We try to answer this question in **chapter 3.3** and we study how tumour pigmentation and chromosome status are related. Our data show that not only eye colour but also loss of chromosome 3 influence tumour pigmentation.

We next turn to potential therapeutic targets for UM (**chapter 4**). We first review the literature regarding MITF, which is the master regulator of many genes that regulate melanin synthesis (**chapter 4.1**), and then analyse the relation between MITF and clinical factors in our Leiden clinical dataset (**chapter 4.2**). We similarly study PRAME, a protein that has been identified as a prognostic factor but also as a potential target in immunotherapy (**chapter 4.3**).

New therapies are often tested on cell lines, as a first step. Cell lines are also used in the next step of pre-clinical research: animal models. It is therefore crucial to characterise and study the cell lines used in experiments, in order to interpret the data correctly. In **chapter 4.4** we study the differences between two cell lines that lack typical UM mutations and those that do have a *GNAQ* or a *GNA11* mutation.

The different studies reported here may help to get a better insight into the biology of UM and the role of different genes in the development of UM and their metastases.

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