UVEAL MELANOMA

from basic science to patient outcome

Jackelien van Beek

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Jackelien (Jacoba Gertrudis Maria) van Beek
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CHAPTER 1

INTRODUCTION

CHAPTER 1.1

General introduction

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In: Dr. April Armstrong. *Advances in Malignant Melanoma - Clinical and Research Perspectives*, 1st edn. Publisher: Intech, 2011: 137-158.

INTRODUCTION

Ocular melanoma is a malignant tumour in the eye and comprises 3% of all melanomas. It is a rare and diverse disease that includes uveal, conjunctival, and orbital melanoma. Among adults of European and American descent, uveal melanoma (UM) is the most common cause of primary eye cancer. UM develops from melanocytes of the uvea that originate from neural crest cells. During embryogenesis, neural crest cells migrate to the neural tract where they differentiate into melanocytes. Approximately 90% of UMs derive from the choroid, with only 6% deriving from the ciliary body and 4% from the iris (**Figure 1**).[1] UMs are generally pigmented, but one-fourth are relatively non-pigmented or amelanotic (**Figure 2**). Choroidal melanoma can develop into two different directions: towards the vitreous or outwards, through the underlying sclera. Having broken through Bruch's membrane, into the retina and vitreous, UMs achieve a characteristic shape, which is even pathognomonic, like a mushroom or collar button. Small melanomas can appear flat or dome-shaped. Posterior uveal melanomas (ciliary body and choroidal) have a strong tendency to metastasize hematogenous to the liver and are associated with high mortality rates. Despite primary treatment, approximately 50% of the patients develop metastatic disease, which is often fatal within one year. Unfortunately, overall survival has not improved over the past 30 years.[2] The primary tumour may show favourable or non-favourable histopathological and molecular genetic features related to the prognosis. These prognostic features are important and could lead to more targeted and personalised therapy.

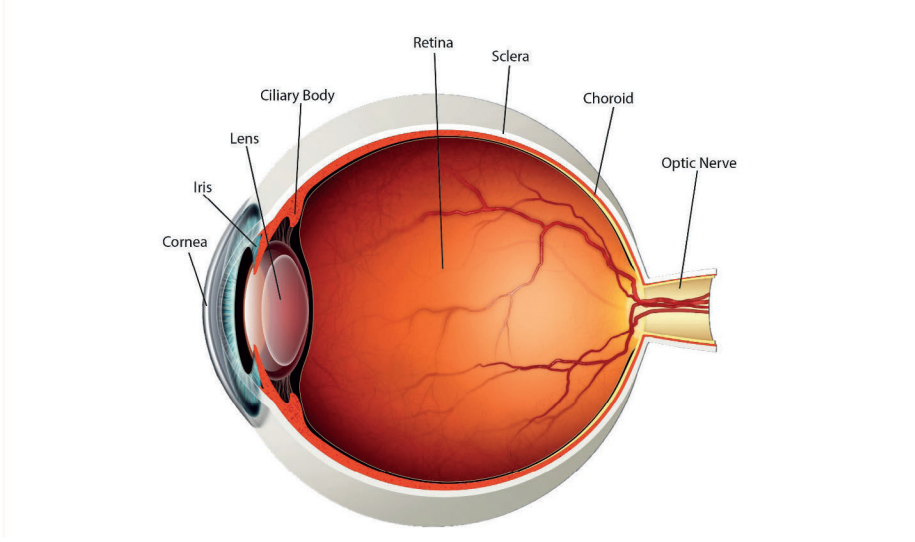

Figure 1. Schematic representation and cross-section of the eye. (Source: Shutterstock)

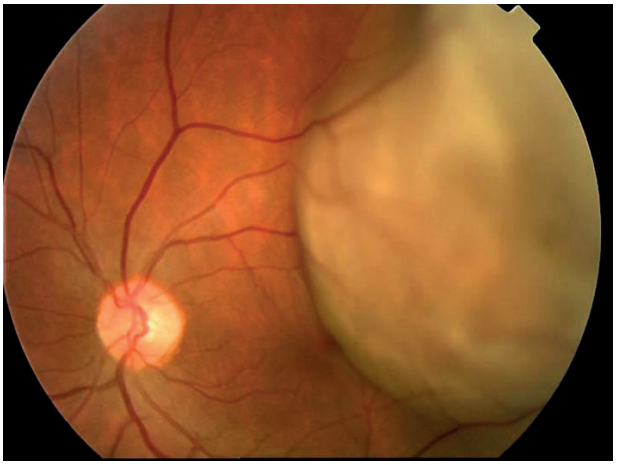

Figure 2. A large amelanotic uveal melanoma leads to a visual field defect.

Epidemiology

The incidence of UM ranges from 4.3 to 10.9 per million per year and has remained stable for the past fifty years.[3] This absolute number is much lower than the incidence rate of 171.6 per million for cutaneous melanoma.[4] The rarity of UM suggests that uveal melanocytes seldom develop into melanoma. However, when comparing the incidence of UM as related to the small surface area of the choroid with cutaneous melanoma and the much larger surface area of the skin, the numbers change. For instance, in a large study from the United States, the incidence of cutaneous melanoma was estimated at 153.5/million persons per year;[5] thus, with an approximate skin area of 1.7 m^2 for a human adult,[6] the incidence of cutaneous melanoma can be estimated at 90 per million m^2 skin per year. In comparison, with a choroidal area approximating the retinal area of 1100 mm,[2 7] the incidence of choroidal melanoma of 4.3/million persons per year [5] can be estimated at 1955 per million m^2 choroid per year. This is a remarkable 20-fold higher per area compared to melanoma of the skin. Therefore, although rare in absolute numbers, the incidence is high considering the size of the choroid. The median age at presentation is 60 years, and women and men are equally affected.[8] For cutaneous melanoma, 20% of the patients are between 50-59 years old, and the gender distribution is roughly equal (43.5% female and 56.5% male).[9] Among individuals younger than age 50, melanoma is more common among females than males, but after age 50, cutaneous melanoma incidence increases steeply among males while stabilizing for females.[10]

Predisposing conditions

Several phenotypes, such as light iris colour and fair skin, have been significantly associated with a predisposition to UM.[11,12] Of all the parts of the uvea, the iris is most exposed to ultraviolet light, but, because of the filtering effects of the cornea, iris, lens and vitreous only visible light reaches the retinal pigment epithelium (RPE).[13] The ciliary body and choroid are not directly exposed to sunlight. Several epidemiologic and case control studies have investigated the influence of sunlight exposure on UM, the results, thus far, are inconclusive.[14-18] Although an association of neurofibromatosis type 1 (NF1) with UM has previously been suggested,[19,20] other studies showed only an occasional relation between NF (1 gene) and UM.[21,22] Dysplastic naevi, cutaneous melanoma, and ocular and oculodermal melanocytosis (Nevus of Ota) are correlated with an increased risk of UM development.[23-29] In UM patients, ocular and oculodermal melanocytosis are about 35 to 70 times more common.[23,29] UM may also occur as a part of familial syndromes, such as xeroderma pigmentosa, Li-Fraumeni syndrome, and familial breast and ovarian cancer, and represents 0.6% of all UMs.[30] A retrospective study by Singh *et al.* [31] showed that 0.0017% of the primary UM patients were in the setting of familial atypical mole and melanoma syndrome (FAMM). These patients were relatively young with a mean age of 40 years.[31] An important predisposing factor for familial cancers is the highly penetrant BRCA1-associated protein-1 (BAP1) tumour predisposition syndrome (BAP1-TPDS). BAP1 is a scavenger protein that regulates cell cycle, cellular differentiation, and DNA damage response. The BAP1-TPDS, on the other hand, is a hereditary tumour syndrome caused by germline pathogenic variants in BAP1 encoding a tumour suppressor. This syndrome has been associated with an increased risk of developing uveal melanoma, mesothelioma, cutaneous melanoma, renal cell carcinoma, meningioma, cholangiocarcinoma, and cutaneous BAP1-inactivated melanocytic tumours.[32,33]

Clinical presentation and diagnosis

Most UMs are detected during a routine ophthalmic examination. Patients usually have no symptoms at time of diagnosis, and, if there are any, these consist mostly of blurred vision, floaters, photopsias, and visual field loss depending on the location and the size of the tumour (**Figure 2**).[34] Patients seldom present with severe ocular pain, but this can occur secondary to inflammation or to neovascular glaucoma.
Diagnosis of UM is based on a combination of clinical examination with slit lamp biomicroscopy, indirect ophthalmoscopy (**Figure 3a**, **Figure 4a** and **Figure 5a**), and ultrasonography (US) (**Figure 3b** and **Figure 4b**). Choroidal tumours, depending on their location, are diagnosed by dilated indirect ophthalmoscopy and US. UM shows characteristic low to medium internal reflectivity on A-scan US. B-scan US is primarily used to plan therapy, based on the first measurement, and to periodically measure tumour prominence (thickness) and basal diameter for follow-up.[35] The internal structure of the tumour is typically seen as a relatively homogeneous grey

scale, although this pattern is not specifically diagnostic (**Figure 4b**). At the base of the tumour, an acoustically silent zone (called acoustic hollowing) is seen, as well as choroidal excavation and shadowing in the orbit (**Figure 3b**). In suspect cases, fluorescein angiography can be helpful in differentiating melanomas from other diagnoses (**Figure 5b**). Indocyanine green angiography is designed to visualize the choroidal vessels. Optical coherence tomography (OCT) and autofluorescence can also provide additional information, such as identifying subtle changes in the RPE, retina and vitreoretinal interface.[36,37] By means of an OCT, subretinal fluid can be visualized (**Figure 3c** and **Figure 4c**) and quantified and small tumours can be measured, whereas, with fundus autofluorescence, orange pigment can be shown. Additionally spectral domain (SD)-OCT can be useful in the detection of subretinal deposits, preretinal vitreous seeding, and transretinal tumour extension.[38] Vitreous seeding is described as discrete, irregularly spheroidal bodies in the vitreous cavity on SD- OCT.[38,39] OCT angiography is mainly used for retinal imaging, and it detects microangiopathy after radiotherapy. Other imaging techniques, such as magnetic resonance imaging (MRI) and computed tomography (CT) can be of additional value in the differential diagnosis of UM and, moreover, seem more sensitive and more specific than US for detecting extra ocular extension of UM.[40]

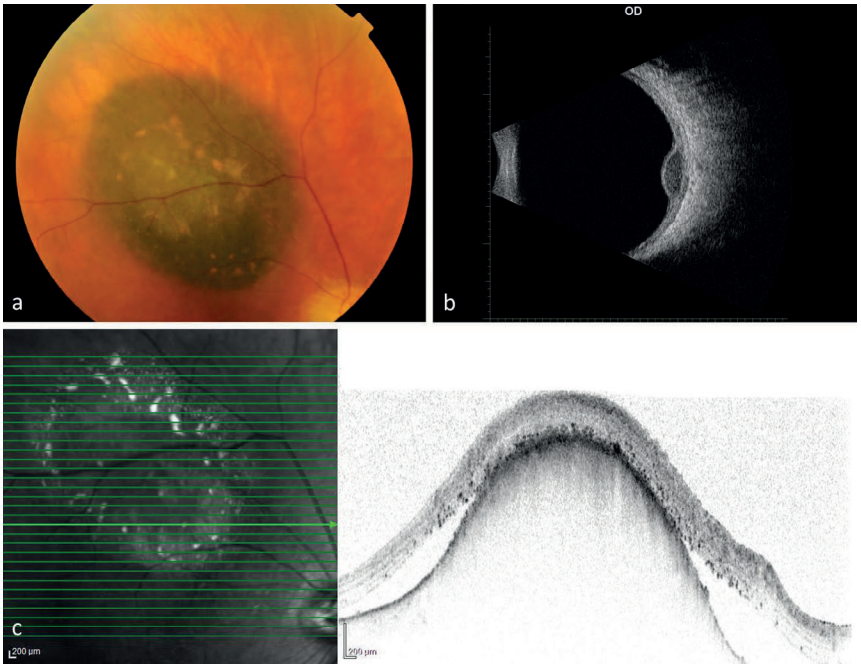

Figure 3a. A dark pigmented uveal melanoma with orange pigment. **Figure 3b.** On B-scan ultrasonography, acoustic hollowing and choroidal excavation is present. **Figure 3c.** Subretinal fluid and retinal pigment epithelial alterations are visible on optical coherence tomography scan at the top of the tumour.

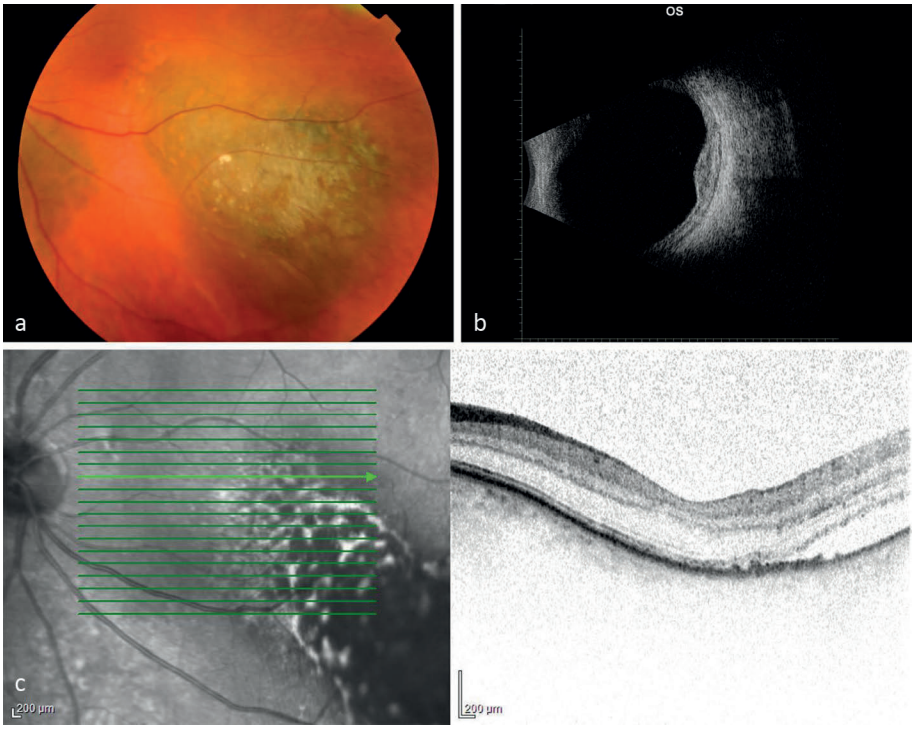

Figure 4a. Pigmented uveal melanoma with orange pigment (lipofuscin). **Figure 4b.** A homogeneous grey scale in the tumour and choroidal excavation on B-scan ultrasonography. **Figure 4c.** Optical coherence tomography of the same tumour with subretinal fluid.

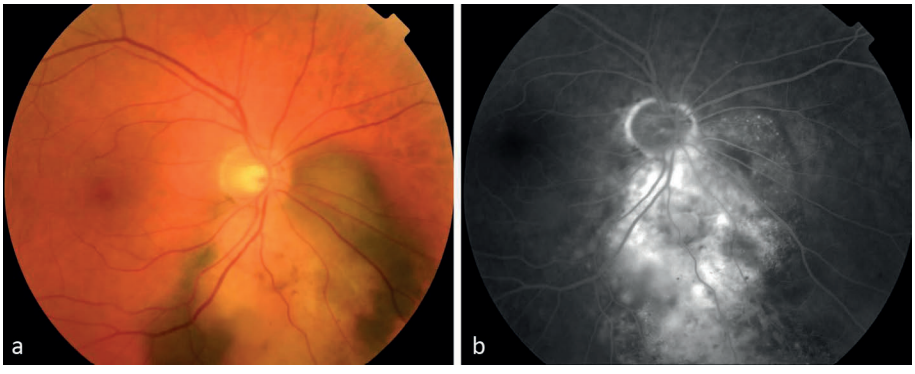

Figure 5a. A partly pigmented and non-pigmented uveal melanoma. **Figure 5b.** Fluorescein angiography with blockage of the background and leaking of fluorescent dye from the bloodvessels.

Clinical classification

UMs are subdivided into different stages (I-IV), of which IV has the worst prognosis. The stages are a combination of the Tumour-Node-Metastases (TNM) category according to the American Joint Committee on Cancer (AJCC) classification. This classification combines tumour size, ciliary body involvement, and extraocular extension to divide UM into these four tumour stages and 17 tumour subcategories (**Table 1**).[41] Because UM does not metastasize to lymph nodes as the majority of solid tumours do, a less detailed staging system is often used devised by The Collaborative Ocular Melanoma Study (COMS) group. The T category (T1-T4) of posterior UM depends on the thickness and the largest basal diameter of the UM in mm. Besides depending on the tumour size to categorise the treatment and assess outcome, other factors influence the prognosis. Damato *et al.* [42] developed an online tool to generate personalised survival curves depending on combined clinical, histological, and genetic predictors (http://www.ocularmelanomaonline.com).

Table 1. AJCC classification of posterior uveal melanoma; Tumour Node Metastases (TNM) stage, T category: T1, T2, T3 and T4 tumours

Tickness (*in mm*)							
> 15.0	4	4	4	4	4	4	4
12.1-15.0	3	3	3	3	3	4	4
9.1-12.0	3	3	3	3	3	3	4
6.1-9.0	2	2	2	2	3	3	4
3.1-6.0	1	1	1	2	2	3	4
≤ 3.0	1	1	1	1	2	2	4
	≤ 3.0	3.1-6.0	6.1-9.0	9.1-12.0	12.1-15.0	15.1-18.0	> 18.0
	Largest basal diameter (*in mm*)						

Source: Adapted from *AJCC Cancer Staging Manual*, 8th ed.[41]

Clinical predictive factors of small melanoma

In general, choroidal naevi have a less than 5 mm basal diameter and are minimal in height (usually < 1.5 mm), although several other defining characteristics of naevi have been proposed. The overall prevalence of choroidal naevi is 4.6- 7.9% in the general Caucasian population,[43-45] and approximately 1 in 8845 choroidal naevi transform into malignant melanomas.[45] Whenever growth of a nevus is measured on US (**Figure 6b**), it is suspected that it could transform into a small melanoma in a relatively short time. On the other hand, benign naevi may also show slow grow. By identifying indicators of potential malignancy which may differentiate naevi from small UM, Shields *et al.* [46] constructed a mnemonic "TFSOM", i.e. "to find

small ocular melanoma". The letters of the mnemonic indicate **T**hickness > 2 mm, subretinal **F**luid, **S**ymptoms, **O**range pigment and **M**argin to the optic disc (of < 3 mm) (**Figure 6a**). Subretinal fluid is the strongest indicator of malignancy.[47] Orange pigment is caused by an accumulation of lipofuscin within the RPE. Tumours with no, one, or more than two factors mentioned above (TFSOM) have 4%, 36% or > 45% chance of growth, respectively.[48]

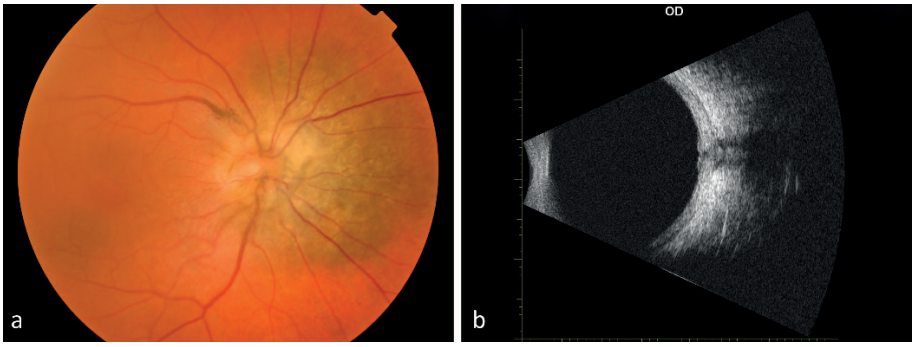

Figure 6a. Juxtapapillary naevus, barely elevated, with margin located < 3 mm to the optic disc in the right eye of a 72-year-old man. **Figure 6b.** High reflectivity on B-scan ultrasonography.

Clinical prognostic factors

Age and location of the tumour are well-known clinical prognostic factors. Older patients tend to have a worse prognosis.[49] In patients with larger tumours, tumours that ruptured through Bruch membrane, and in patients who have developed metastasis, the tumours are significantly more often located anterior to the equator.[50] Patients with iris melanoma have a lower chance of developing metastases than ciliary body melanoma or choroidal melanoma with ciliary body involvement. Extra ocular extension is growth of the tumour by different routes through the sclera into the periocular tissues. It may be clinically visible as a brown mass if located in the anterior part of the eye or visible using US or MRI if located in the posterior part of the eye. The B-scan can identify possible extra ocular extension as an empty area behind the sclera. Extra ocular extension is associated with increased mortality because it is associated with increased tumour malignancy and, in case of posterior located tumours, with advanced disease.[51] Eventually, the most important clinical prognostic factor is tumour size and this is often used for selection of the treatment. Kaplan-Meier estimates of death at 5 years was 4% for category T1, 8% for category T2, 19% for category T3, and 30 % for category T4; at 10 years it was 8% for T1, 13% for T2, 27% for T3, and 43% for T4; at 20 years it was 11% for category T1, 24 % for category T2, 36% for category

T3, and 51 % for category T4 ($p < 0.001$).[52] Each millimetre increase in tumour thickness seems to increase the risk of metastases by approximately 5%.[1] This supports the model of the tumour doubling time of melanoma and its related metastasis. The model suggests that hematogenic seeding and micrometastasis already occur several years before diagnosis of the primary tumour.[53] Again, this emphasizes the importance of identifying small melanoma and reducing the risk of metastases.

Histopathologic factors

Currently, three histopathological uveal melanoma types, spindle, epithelioid, and mixed cell type are defined based on the Callender classification.[54,55] Haematoxylin and eosin (H&E) staining is used to differentiate between cell types (**Figure 7**). Spindle type A cells are spindle-shaped, with a linear infold in the nucleus, while spindle type B cells have round to oval nuclei, prominent nucleoli, and indistinct cell borders (**Figure 7b**).[56] In general, tumours containing spindle cells grow slowly and might be associated with better prognosis. Intermediate celles (or small epithelioid cells) are larger than spindle B cells and are intermediate between spindle type B and epithelioid cells. Epithelioid cells have more polygonal cytoplasm and contain eccentric placed large pleomorphic nuclei and prominent eosinophilic nucleoli (**Figure 7c**). UM consisting of faster growing epithelioid cells, have a more aggressive behaviour, and are therefore associated with poor clinical outcome. The mixed-cell type melanoma has a variable proportion of spindle B cells and epithelioid cells with a minimum of 10% of any one type.[57] Other inter-tumour factors, such as the presence of certain extracellular matrix patterns (three closed loops located back-to-back identified by Periodic-acid Schiff (PAS) staining without Haematoxylin counterstaining using a green filter) and increased mitotic figures (number of mitoses per 50 high-power fields equal to 8 mm^2) can both provide additional adverse prognostic information.[58,59] Other histological features associated with mortality and metastases are the mean diameter of the ten largest nucleoli, the degree of pigmentation, the presence of inflammation, and tumour necrosis.[60] Extrascleral extension or spread by perineural, perivascular, intravascular, or direct scleral invasion is correlated with a worse prognosis, especially when the orbital fat resection margin is positive.[61] Extraocular spread of UM may provide access to subconjunctival lymphatics and, moreover, has been hypothesized as promoting intraocular lymphangiogenesis through recruitment.[62] The question remains: are intraocular lymphatic vessels present in eyes with uveal melanoma and extrascleral extension?

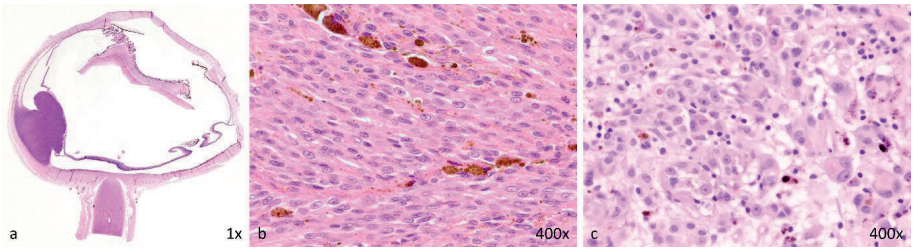

Figure 7a. Haematoxylin and eosin staining of formalin fixed and paraffin embedded eye sample with a typical mushroom shaped melanoma. **Figure 7b.** Uveal melanoma tissue with spindle cell type characterised by elongated nuclei. **Figure 7c.** Uveal melanoma tissue with epithelioid cells containing large pleomorphic nuclei and prominent eosinophilic nucleoli.

Genetic classification

Somatic human cells have 46 chromosomes, arranged in two sets of 23. All cancers begin when one or more genes in a cell mutate. Usually, multiple mutations accumulate over a lifetime, and, depending on the type of gene and where in the gene the changes occur, the outcome may be beneficial, harmful, or neutral to the organism as a whole.

Cytogenetic studies in solid tumours have been a greater challenge than in haematological malignancies since metaphase chromosome spreads of good quality are more difficult to obtain. Solid tumours frequently have highly complex chromosome alterations and are more heterogeneous. Despite this challenge, UM has been well studied with different techniques since the late 1980s. Over the years, we have learned that the majority of UMs often contain non-random chromosomal anomalies on either the short arm (p) and or long arm (q) of chromosomes 1, 3, 6 and 8 (**Figure 8a**), and as such, can serve as prognostic markers.

Cytogenetic and molecular techniques in UM research

UMs are quite suitable for cytogenetic analysis because of their relatively simple karyotype. For the detection of smaller abnormalities, techniques such as FISH (**Figure 8b**), comparative genomic hybridization (CGH), or quantitative polymerase chain reaction (qPCR) based techniques are necessary.

After completion of the human genome project, genome-wide DNA assays have become available. With the development of Next Generation Sequencing (NGS) technologies, the genome can be selectively analysed at base pair level. Genome-wide mutation analysis of tumour samples led to the discovery of mutations in a subset of genes, such as *GNAQ* and *BAP1,* in UM.

The genetic evolution of UMs continues to progress moderately as they progress from primary tumour to metastases. Indeed, even after successful treatment of the primary tumour and after a long latency period, metastases may develop mainly in the liver.

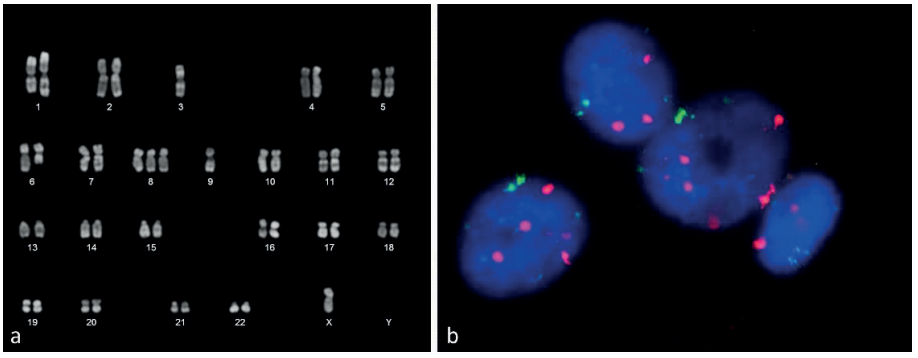

Figure 8a. Example of a karyogram showing monosomy 3 and trisomy of chromosome 8. **Figure 8b.** FISH analysis of a tumour demonstrates 1 signal for the probe on centromere 3 (green signals) and 3 to 4 signals of the probe on centromere 8.

Chromosomal anomalies

Chromosome 3

Monosomy of chromosome 3 is observed in approximately 50% of the cases of UM and is strongly associated with clinical and histopathological prognostic factors and with metastatic death.[63-65] Prescher and associates were the first to find a strong correlation between loss of chromosome 3 and a poor patient outcome.[66] Since then, several groups have confirmed the prognostic value of monosomy 3.[67-70] It is assumed that loss of chromosome 3 is a primary event, as it often occurs with other chromosomal aberrations in UM, such as 1p loss and gain of 6p and 8q.[71] Mostly, one entire copy of chromosome 3 is lost, although in some cases, isodisomy of chromosome 3 is acquired.[72-74] Partial deletions or translocations have rarely been described on this chromosome, making it difficult to locate putative tumour suppressor genes. However, a mutation in the *BAP1* gene, located on chromosome 3, has been identified in UM, and this gene plays an important role in the tumour progression.[75] This gene will be discussed in more detail later in this chapter.

Chromosome 8

Abnormalities in chromosome 8, and in particular gain of 8q or an isochromosome 8q, are thought to be a secondary event in UM as variable copy numbers can be present in one melanoma.[76,77] Gain of chromosome 8q is frequently found in tumours that also have loss of chromosome 3, and this abnormality is associated with an increasingly worse patient outcome.[70-72] A SNP array analysis with this chromosome status is depicted in **Figure 9**. The relationship between the percentages of aberrant copy numbers within UM cells and patient outcome has been investigated. These studies found that a higher percentage of monosomy 3 and chromosome 8q gain in primary UM cells shows a strong relation with poor disease-free survival compared to low percentage aberrations.[78,79]

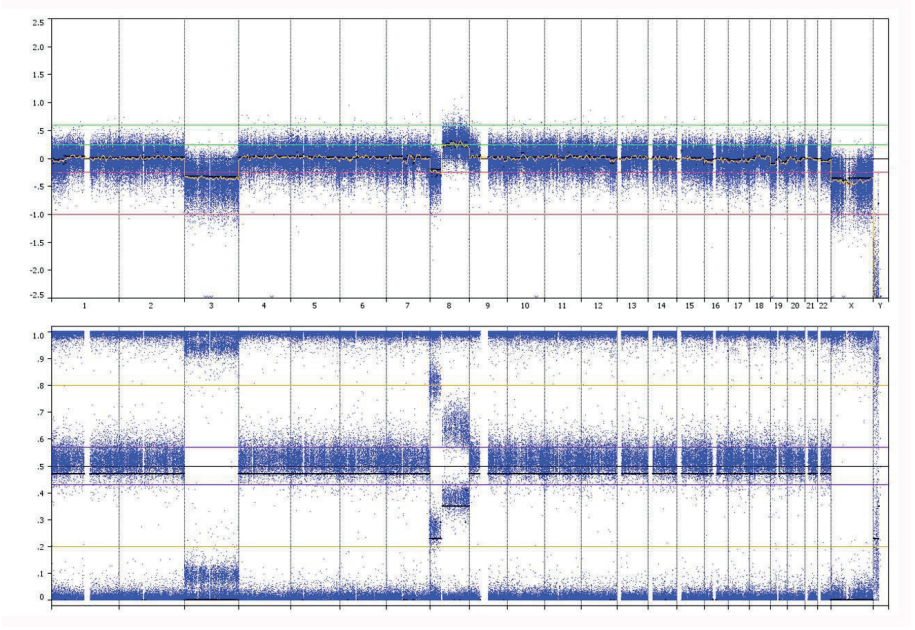

Figure 9. Single nucleotide polymorphism (SNP) array of an uveal melanoma. The upper panel shows loss of chromosome 3, partial loss of chromosome 8p and gain of chromosome 8q. The lower panel depicts the B-allele frequency representing allelic imbalance at these chromosomes.

Chromosome 6

Rearrangements on chromosome 6 affect both arms of the chromosome, resulting in deletions of 6q and gain of 6p. The relative gain of chromosome 6p can occur either through an isochromosome of 6p or a deletion of 6q. Tumours with gain of 6p are thought to be a separate group within UM with an alternative genetic pathway to carcinogenesis, since gain of 6p is frequently found in tumours with disomy 3.[69,80,81] Although aberrations resulting in a relative increase of 6p have been found to be related with both a longer and decreased survival [70,72], the effect of chromosome 6 aberrations on patient outcome is not conclusive.[82]

Chromosome 1

In UM, this region on 1p is frequently affected, giving rise to a deletion of 1p. Tumours with concurrent loss of chromosome 1p and 3 are at higher risk of metastasis.[83,84]

Aberrations on other chromosomes have been explored, such as chromosome 9p21 [73], chromosome 11q23 [68], chromosome 18q22 [2,85], and chromosome 16q.[67,86] The impact on the prognosis, however, remains unclear due to contradictory findings.

Candidate genes

With the introduction of high throughput sequencing techniques, it became possible to use DNA extracted from the tumour to identify those genes involved in tumorigenesis (such as *GNAQ/GNA11)* or in secondary driver genes (such as *BAP1, SF3B1* and *EIF1AX)* that are associated with the progression of UM towards metastasis.

GNAQ and GNA11 gene

With the discovery of activating *GNAQ* (guanosine nucleotide-binding protein Q polypeptide on chromosome 9q21.2) and *GNA11* (guanosine nucleotide-binding protein alpha-11 on chromosome 19p13.3) mutations, new light has been shed on the MAPK (mitogen-activated protein kinase) pathway. A subsequent study reported that 83% of UM samples harboured Gα-protein mutations (*GNAQ* or *GNA11* mutations), affecting specific regions on either exon 4 or 5 (codon R183 or Q209, respectively) in a mutually exclusive pattern.[87] Other studies have confirmed these results, and *GNAQ*/*GNA11* mutations have been found in nearly all UM cases.[88,89] When there were no mutations in *GNAQ* or *GNA11*, recurrent mutations in *CYSLTR2* (Cysteinyl Leukotriene Receptor 2) and *PLCB4* (Phospholipase C Beta 4)

were found in some of these cases.[90,91] The oncogenic conversion of *GNAQ or GNA11* is suggested to be the cause of constitutive MAPK pathway activation. These mutations also activate the YAP (Hippo-Yes-associated protein) or TAZ (transcriptional coactivator with PDZ-binding motif) pathway.[92] There is no relation between *GNAQ* and *GNA 11* mutations and prognosis as these mutations are found in nearly all UM and in benign blue naevi.[88,93] Hence, the presence of Gα-protein mutations in tumours at all stages of malignant progression and in melanocytic lesions of the choroid suggests that they are early events in UM.[89,93]

BAP1 gene

Secondary driver genes, such as *BAP1, SF3B1* and *EIF1AX* , are strongly associated with prognosis.[94,95] Whole exome genome sequencing has led to the discovery of the BRCA1 associated protein 1 (*BAP1*) gene in UM.[75] *BAP1,* a nuclear localized enzyme, has deubiquitinating activity and is involved in several biological processes, including regulation of cell cycle and cell growth, chromatin dynamics, and DNA damage response.[96] *BAP1* is located on chromosome 3p21.31-p21.2 and is thought to be a tumour suppressor gene.[97] Inactivating somatic mutations were found in 84% of the metastasizing UMs.

SF3B1 and EIF1AX

SF3B1 (splicing factor 3B subunit 1), a hot spot mutation in a splicing gene, has been reported in approximately 25% of the UM cases, with these mutations being reported at R625, K666 and K700.[98] *SF3B1* mutated tumours are associated with an increased (late onset) metastatic risk compared to UM without *SF3B1* mutations and are related to disomy 3 tumours.[99] The *SF3B1* gene, located on chromosome 2q33, is the most frequently mutated spliceosome gene, and spliceosome mutated UM show different prognosis, pathogenesis, genetics, and epigenetics.[100]

*EIF1AX (*eukaryotic translation initiation factor 1A, X-linked) mutations are found in approximately 20% of the UM.[95] *EIF1AX,* located on chromosome Xp22, is involved in the initiation of gene translation in eukaryotic cells; missense and nucleotide deletion variants alter the N-terminal tail codons of *EIF1AX*. *EIF1AX* mutated cases, compared to those without *EIF1AX* mutations, showed a significant increase in disease-free survival.[101]

Gene expression profiling and epigenetics

Recently, four subsets of UM have been defined with their corresponding low, intermediate, or high risk of developing metastasis.[102] Those four types (Subset A, B, C, D) differ in genetic aberrations, methylation pattern, gene expression profile (GEP), and immunological characteristics.[103] Using gene expression profiling, UMs can be classified into two classes of tumours that correspond remarkably well with the ability of the tumour to metastasize. This 15-gene expression profile test based on mRNA expression analysis has been clinically validated and widely used and can strongly predict metastatic death and outperform other clinical, histopathological, and cytogenetic prognostic indicators.[104-107] Class 1A tumours have a low risk of metastasizing, Class 1B tumours have an intermediate risk, whereas Class 2 tumours have a high risk of developing metastasis.[108] In addition to GEP, other chromosomal and molecular markers have been used to estimate prognosis of UM. According to The Cancer Genome Atlas (TCGA) classification for UM, the subtypes of the tumours mentioned above can be subdivided in Subset A, B, C, or D, with the most advanced tumours in Subset D.[102] The key mutation in Subset A is *EIF1AX,* in Subset B *SF3B1*, and in Subset C and D *BAP1*.

- Subset A: UM with disomy of chromosomes 3 and 8, partial or total gain chromosome 6p, Class 1 mRNA GEP.
- Subset B: UM with disomy of chromosome 3 and chromosome 8q partial gain, gain chromosome 6p, Class 1 mRNA GEP.
- Subset C: UM monosomy of chromosome 3 and chromosome 8q gain, normal chromosome 6p, Class 2 mRNA GEP.
- Subset D: UM with monosomy of chromosome 3 and multiple chromosome 8q copies, normal chromosome 6p, Class 2 mRNA GEP.

Additionally, epigenetic alteration, such as changes in microRNAs (miRNA) and long non-coding RNAs (lncRNA), also plays a role in the development and metastasis of UMs.[109,110] MiRNA and lncRNA, unlike mRNA, do not produce proteins; however, they play a role in the regulation in transcription processes. MiRNAs are small, single-stranded RNA and the downstream effect of aberrant miRNA expression is the deregulation of several oncogenic pathways.[111]

Robertson *et al.* [102] assessed the methylation profile in UM. The *BAP1* group in monosomy 3 UM showed a unique global DNA methylation pattern compared to the disomy 3 UM, which suggests that aberrancy in *BAP1* could lead to a metastasis-prone DNA methylation state. In the disomy 3 group, a distinct methylation pattern was observed between the *EIF1AX* group and the *SF3B1* group. The different methylation profile seen here could contribute to the associated prognosis of both

groups.[102] This and the different miRNA patterns could be promising therapeutic targets and an interesting biomarker for metastatic risk.

Metastases

Irrespective of the primary treatment of UM, nearly half of the patients develop metastases. UM spreads haematogenous, with a high tendency to metastasize to the liver in 90-95% of the patients. Besides the liver, metastases can occur in lymph nodes, lungs, bones, skin, and the brain. One explanation for the development of distant metastasis years after treatment of the primary tumour is the presence of circulating tumour cells at time of the initial diagnosis.[112] In other words, the disease is often already disseminated at the time of the diagnosis. In the case of liver metastasis, the prognosis is poor with a median survival of approximately 8 months.[113] Although there are no therapeutic options for metastatic UM that improves survival or quality of life, the following methods can be used for screening liver metastasis: liver function tests (gamma-glutamyl transpeptidase (γGT) and lactate dehydrogenase (LDH)) from the blood, liver imaging with US, CT or MRI. Although screening annually or semi-annually for liver metastasis by liver function tests is widely used, there are reports of disseminated liver metastases and normal liver function tests.[114,115] Patients have a 97.5% chance or more of having no metastasis in the case of normal liver function tests because of the high negative predictive value. However, isolated or combined liver function tests for aspartate aminotransferase (AST), alanine transaminase (ALT), γGT, LDH, and phosphatidic acid (PA) are not indicated for the detection of early liver metastasis.[116]

Treatment of primary UM

The choice of treatment of UM depends on several factors: the location and size of the tumour, the secondary effects of the tumour on the eye, the status of the fellow eye, and the patients' preference (**Table 2**). Until the late eighties, the only available treatment was enucleation of the affected eye. Currently, there are eye-preserving treatment options, such as radiotherapy (local irradiation techniques). If the tumours are larger, advanced and, in particular, if there is evidence of extra ocular extension, enucleation is advised.[117] Even though enucleation is sometimes required, eye-preserving approaches have shown to be equally successful regarding overall survival and metastasis-free survival.[118,119]

Table 2. Treatment modalities for uveal melanoma

Treatment	Indications	Adverse side effects	Local tumour control
Brachytherapy	Thickness < 7 mm, location accessible for plaque.	Radiation retinopathy, maculopathy, optic neuropathy, cataract, vitreous haemorrhage, neovascular glaucoma	82-98% [120-123] 5-years: 73.0-98.0%[123-126] 10-years: 75.7%[124]
Stereotactic radiotherapy	LTD < 16 mm and tumour thickness < 12 mm.	Radiation retinopathy, maculopathy, optic neuropathy, cataract, vitreous haemorrhage, neovascular glaucoma, dry eye	84-98%[127-129] 5-years: 82.0-95.9%[130-132] 10-years: 90.2-92.6%[130,131]
Proton beam radiotherapy	LTD < 20 mm and tumour thickness < 12 mm.	Radiation retinopathy, maculopathy, optic neuropathy, cataract, vitreous haemorrhage, neovascular glaucoma, dry eye	90-99%[133-136] 5-years: 92.7-98.9%[131,136-138] 10-years: 88.1-95.7%[131,138]
Photodynamic therapy	Amelanotic small tumours, often neo-adjuvant before brachytherapy. Role for pigmented UM unclear.	Scleritis, vascular occlusion, choroidal atrophy, intravitreal haemorrhage, exsudative retinal detachment	80-89%[139]
Endoresection	Thickness > 6-8 mm, more posterior located. Only as primary treatment when radiotherapy was considered to cause optic neuropathy or maculopathy.	Intra- and postoperative complications, such as vitreous haemorrhage, hyphema, subretinal haemorrhage, cataract, retinal detachment with or without proliferative vitreoretinopathy, choroidal detachment, corneal edema, macular edema and elevated IOP.	94.2-96.9%[140-142] 5- and 10-years: 96.3%[143]
Exoresection	Large UM; Thickness > 8 mm; with ciliary body and/or iris involvement < 3 clock hours. Prognostic Fine-needle aspiration biopsy.	Intra- and postoperative complications, such as vitreous haemorrhage, hyphema, subretinal haemorrhage, cataract, retinal detachment with or without proliferative vitreoretinopathy, choroidal detachment, corneal edema, macular edema and elevated IOP.	May be beneficial to primary radiation modalities to reduce radiation-induced side effects
Enucleation	LTD > 16 mm with thickness > 2 mm or thickness > 10 mm regardless of LTD. Advanced tumours with extraocular extension.	Pain, chemosis, superior sulcus deformity, scarring of the socket, exposure/extrusion and loss of the orbital implant	If extraocular extension is present, orbital recurrence can occur in 10%

LTD= largest tumour diameter

IOP= intraocular pressure

UM= uveal melanoma

Brachytherapy

Brachytherapy is the most common modality for the treatment of small and medium-sized UM by local irradiation. During brachytherapy, a radioactive shield or plaque is placed on the eye precisely at the location of the tumour during surgery. Currently, the plaques that are the used are ruthenium-106, iodine-125, palladium-103, iridium-192, strontium-90 and cobalt-60 (now obsolete). The time the shield remains on the eye depends on the isotope used. When the tumor apex dose between 70-100 Gray is reached is reached, the shield is removed. Brachytherapy can be used in combination with other methods of treatment of UM, such as local resection or transpupillary thermotherapy.[144,145] Significantly, local tumour control with plaque radiotherapy has provided an overall survival comparable to enucleation. Local recurrences after brachytherapy are reported to be between 4-28%, depending on the size of the tumour and the follow-up time.[146-152] Radiation-induced complications include radiation retinopathy, radiation maculopathy, radiation optic neuropathy, neovascular glaucoma, cataract, retinal detachment, vitreous haemorrhage as well as tumour recurrences.[153-155] In 10–22% of the patients, radiation-induced side effects lead to secondary enucleation.[150,156-164] However, the treatment of most of these side effects can improve with anti-VEGF (vascular endothelial growth factor) or steroid intravitreal injections, laser therapy, and resection of the tumour to prevent the 'toxic tumour syndrome'. Toxic tumour syndrome may occur after radiation treatment of a choroidal melanoma and may involve the release of inflammatory cytokines, exudation from irradiated and incompetent vessels, and release of VEGF from irradiated ischemic tissue.[134,165]

Proton beam radiotherapy

Heavy particle radiation with positive charged particles (protons or helium-ions) enables treatment of small-, medium- and large-choroidal melanomas. Proton beam radiotherapy (PBR) is available in a growing number of centres in Europe and even recently available in Delft. Before radiation, clips are sutured to the sclera around the base of the tumour. With PBR, radiation is delivered homogenously to the tumour with the dose rapidly falling to zero at a short distance of the tumour.[166] The local recurrence rate for proton beam irradiation is similar to brachytherapy and is usually around 5% at 10 years.[147,152] Secondary enucleation is performed in 5-16% of patients either because of local recurrence or side effects.[133,167,168] The following radiation-induced adverse side effects have been reported: maculopathy, retinopathy, optic neuropathy, cataract, glaucoma, vitreous haemorrhage, retinal detachment, and dry eye.[61,169-171]

Stereotactic radiotherapy

Stereotactic radiotherapy (SRT) is suitable for small and medium sized UMs and can be performed using a gammaknife, a CyberKnife or a linear accelerator. The advantage of SRT is that it does not require surgical procedures to determine the tumour localization and dimensions. The tumour is depicted with CT and/ or MRI, while a mask is fixated on the patient's face and an infrared tracking system records eye movements. In concordance with proton beam irradiation, radiogenic-side effects are also reported after SRT. Adverse side effects, such as radiation maculopathy, retinopathy, optic neuropathy, and neovascular glaucoma are responsible for the majority of secondary visual loss and secondary enucleations in 3-16% of cases after SRT.[127,128,172-174] In patients treated with SRT, local tumour control rates have been reported to be between 90% at 5 and 10 years after treatment and 94-100% with a mean follow-up of approximately 25 to 37 months.[127,128,172,175,176]

Photodynamic therapy

Photodynamic therapy (PDT) is not often used as treatment for UM.[177] Although amelanotic (small) tumours can primarily or additionally be treated in this manner.[178] In PDT, a laser activates Verteporfin, a photosensitive dye, to induce tumour necrosis and apoptosis.

Surgery

Local resection (endoresection and exoresection) of UM aims to conserve the eye and preserve vision. The tumour can be removed in several ways: through the vitreous and retina with a vitreous cutter (endoresection), or through a scleral opening (exoresection). Examples of exoresection includes cyclochoroidectomy, and choroidectomy. Endoresection as well as exoresection can be used as a primary procedure after radiotherapy as a treatment option for preventing recurrences or preventing toxic tumour syndrome. An advantage of local resection is that eyes that would otherwise be enucleated can be preserved. Often, radiotherapy is administered prior to local surgery, or surgery is followed by treatment with brachytherapy because of the apprehension of tumour seeding. However, tumour recurrence and the rate of metastasis appear not to be higher after endoresection.[143] Additionally, relatively large tumour samples are available for prognostication using this procedure.[146,179,180]

Treatment of liver metastases

Unfortunately, when metastatic disease occurs, there is no curative or standardized treatment. Metastases mostly spread to the liver, and death often follows within

one year after systemic symptoms occur. Metastatic disease develops mostly in patients who have tumours that show chromosome 3 loss, *BAP1* loss, or a Class 2 gene expression profile (group C and D).

For liver metastases, several locoregional techniques are available, for example, immune-embolization, chemoembolization, isolated liver perfusion, and hepatic intra-arterial chemotherapy. Systemic treatment options, such as intravenous chemotherapy and immunotherapy, do not seem to give promising results or survival benefit.[181] In highly selected patients, surgical resection of liver metastases can improve survival within a few months. Operating on patients at the time of diagnosis from the primary tumour to liver metastases of > 24 months, ≤ 4 liver metastatic lesions, and absence of 'miliary' disease (multiple, diffuse, millimetre-sized, dark punctuate lesions on CT) is associated with prolonged survival.[182] Patients with complete liver resections had a median survival of 27 months versus patients with incomplete liver resection of 14 months.

Currently, targeted systemic therapies in metastatic UM are under investigation. Clinical trials for patients with advanced metastatic disease are based on the protein kinase C-MAPK (PKC-MAPK) signaling pathway inhibition, immunotherapy, and epigenetic therapies (www.clinicaltrials.gov). In contrast to cutaneous melanoma, kinase inhibitors and immune checkpoint inhibitors are usually ineffective in patients with metastatic uveal melanoma. Currently, promising preliminary results of the immunotherapy with Tebentafusp, an anti-gp100 ImmTAC molecule are being presented at scientific meetings.[183] Tebentafusp shows promising clinical activity in patients with UM metastases and survival rates seem to be prolonged.

Clinical trials are needed for this rare disease and, hopefully, an UM specific treatment based on immunological or mutational content will lead to improved patient survival.

Quality of life

As in all patients diagnosed with cancer, there has been increasing emphasis on the quality of life (QOL). QOL is a highly subjective and dynamic process, subject to changes in life and life events. Treatment of UM seemed to reduce QOL at least in the first three years after diagnosis.[184,185] Irradiated patients had a higher role functioning score than enucleated patients, suggesting better functioning with regard to daily tasks, hobbies, and work. Enucleation patients, on the other hand, had greater problems with cosmetic appearance.[186] Uncertainty about prognosis, changes in physical appearance, and visual problems all contribute to worry and stress in uveal melanoma patients.

CHAPTER 1.2

Scope and outline of this thesis

This thesis aims to evaluate not only clinical, histopathological and molecular genetic prognostic factors in uveal melanoma, as well as the treatment outcome and the quality of life of uveal melanoma patients after treatment.

One of the first questions that may arise in a patient faced with a diagnosis of cancer is, what is my prognosis? Prognostic factors are certain features available at the time of diagnosis that can be used to predict disease outcome and tumour recurrence. Optimizing cancer treatment based on prognostic factors plays a crucial role in the management of uveal melanoma (UM). The prognosis of UM depends on several established clinical, histopathological and molecular genetic factors, such as patient age, tumour diameter, extrascleral extension, ciliary body involvement, epithelioid cell type, and chromosomal aberrations including loss of chromosome 3 (monosomy 3) and gain of chromosome 8q. Moreover, locoregional anatomy determines the fate of the patient with UM.

Part 1 of this thesis is based on the prognostic value of the histopathological characteristics, locoregional anatomic factors and chromosomal alterations in UM presented in **Chapters 2** to **6**. In **Chapter 2**, we describe the prognostic value of extraocular extension (EXE) in relation to monosomy 3 and gain of chromosome 8q in UM. The peculiar locoregional anatomy of the orbit with an absence of lymphatic vessels is highlighted in **Chapters 3** and **4.** The question is whether orbital lymphatics exist and whether they play a role in EXE. Never before has a panel of five immunohistochemical markers been proposed to identify lymphatic vessels with greater accuracy. In **Chapter 3**, UM tissue with EXE was analysed with these immunohistochemical markers. Lymphatic vessels are derived from venous endothelial cells during embryogenesis. For the development of lymphatic vessels, we focus in **Chapter 4** on the expression of lymphatic markers in the retrobulbar intraconal orbital tissue of the fetal and of the developing and the adult eye. **Chapters 5** and **6** describe the role of molecular genetic alterations in relation to disease-free survival and progression of UM.

Part 2 of this thesis illustrates an overview of the clinical factors influencing the treatment outcome and the quality of life of UM patients in **Chapters 7** to **9**. Depending on the tumour size and location, the status of the other eye and patients' choice, UM will be treated with eye-sparing techniques, such as fractionated stereotactic radiotherapy (fSRT), proton beam therapy (PBR), brachytherapy and occasionally photodynamic therapy. The non-eye-sparing treatment is removal of the eye (enucleation). The local tumour control and adverse side effects of UM patients treated with fSRT with at least a follow-up of five years are presented in **Chapter 7.** These results were compared in **Chapter 8** in a matched cohort with the radiogenic side effects of UM treated with PBR in Liverpool, UK. Ultimately, what matters to cancer patients is their quality of life. In **Chapter 9**, the quality of

life and visual functioning are evaluated in a prospective study with questionnaires of UM patients after fSRT or enucleation.

Finally, **Chapter 10** describes the most important findings and provides suggestions for improvement of treatment strategies and future research.

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PART I

PROGNOSTIC VALUE OF HISTOPATHOLOGIC CHARACTERISTICS AND CHROMOSOMAL ALTERATIONS IN UVEAL MELANOMA

CHAPTER 2

THE PROGNOSTIC VALUE OF EXTRAOCULAR EXTENSION IN RELATION TO MONOSOMY 3 AND GAIN OF CHROMOSOME 8Q IN UVEAL MELANOMA

Jackelien van Beek
Anna Koopmans
Jolanda Vaarwater
Johan de Rooi
Dion Paridaens
Nicole Naus
Annelies de Klein
Robert Verdijk
Emine Kiliç

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ABSTRACT

Purpose. To identify the prognostic value of extraocular extension in enucleated uveal melanoma (UM) patients and to correlate extraocular extension to chromosomal aberrations, metastasis-free survival (MFS), and clinico-histopathological risk factors.

Methods. Retrospective study of patients with UM treated with enucleation between 1987 and 2011. Melanoma-related metastasis and death were recorded. Statistical analysis (log-rank test or Cox regression analysis) was performed to correlate MFS with tumor characteristics, extraocular extension, episcleral diameter of the extraocular extension, cell type, extracellular matrix patterns, inflammation, loss of chromosome 3, and gain of chromosome 8q.

Results. In 43 (12%) of 357 patients, extraocular extension was observed. In this subset of patients, we noted a reduced survival of 70 months (105.5 months, $P = 0.010$) compared with patients without extraocular extension (175.8 months). Patients with gain of chromosomal region 8q in UM with extraocular extension had an increased risk of metastatic disease ($P < 0.001$). In multivariate Cox proportional hazard analysis, largest basal tumor diameter ($P = 0.001$), extracellular matrix patterns ($P = 0.009$), episcleral diameter of the extraocular extension ($P = 0.016$), loss of chromosome 3 ($P < 0.001$), and gain of 8q ($P < 0.001$) were independent predictors for MFS.

Conclusions. Larger episcleral diameter of the extraocular extension and additional gain of chromosome 8q in extraocular extension UM correlates to a worse prognosis. MFS is significantly reduced in UM with a large basal tumor diameter, extracellular matrix patterns, loss of chromosome 3, and gain of chromosome 8q.

INTRODUCTION

The age-adjusted incidence of uveal melanoma (UM) is 5.1 per million since 1973.[1] During the past decades, several risk factors have been identified and related to survival. Clinical factors that correlate with poor survival are a large tumor thickness and tumor basal diameter, ciliary body localization, mushroom configuration, and older age.[2,3] The tumor size is of great importance, as each millimeter increase in tumor thickness seems to increase the risk of metastases by approximately 5%.[4] Histopathological risk factors associated with decreased survival are epithelioid cell type, high mitotic activity, presence of extracellular matrix patterns, and extraocular extension.[5-7] UM with epithelioid cells tend to have a more aggressive behavior and are therefore related to a poor clinical outcome. From the known prognostic parameters, the genetic alterations are by far the most strongly associated with metastatic disease. Loss of chromosome 3 or monosomy 3 is observed in approximately 50% of the UMs and is not only associated with clinical but also with histopathological prognostic factors and metastatic death.[8-11] A higher percentage of monosomy 3 leads to a poorer disease-free survival.[12] The same is true for gain of chromosome 8q, and when these abnormalities occur simultaneously, the prognosis is even worse.[13] Van den Bosch *et al.*[12] showed that gradual increase in copy number of chromosome 8q shortened survival. Extraocular extension occurs in 2% to 15% of the UMs.[3,5,14-16] Tumors with extraocular extension are classified in a different subcategory of the TNM classification and are associated with a worse prognosis.[3,14] Moreover, the larger the extension diameter, the shorter the survival will be. The 5-year survival of UM patients with an extraocular extension of 5.1 mm or more is between 18% and 22%.[14,16] Extraocular extension has been correlated with monosomy 3; however, no associations have been found between extraocular extension and chromosome 8q alterations.[5,13] Therefore, the aim of this study was to identify monosomy 3 and gain of 8q as additional risk factors, besides clinical and histopathological factors, in UM with extraocular extension and correlate these with metastasis-free survival (MFS).

METHODS

Patients and Clinical Characteristics

A retrospective study was carried out by the Rotterdam Ocular Melanoma Study group, in patients with a choroidal or ciliary body UM who underwent primary or secondary enucleation from 1987 until 2011. We excluded patients with iris melanoma and cases in which no sufficient tumor material was available to describe the histopathological characteristics of the tumor. The following data were recorded: sex, location of the tumor, date of enucleation, age at time of enucleation, development of metastases, and date and cause of death. If measurements of the

tumor thickness and largest basal diameter from B-scan ultrasonography (US) were not available, we used the tumor's measurements before histological preparation. Tumor measurements obtained before fractionated stereotactic radiotherapy (fSRT) were used if patients had received primary fSRT. Data on extraocular extension was registered during US, surgery, or histopathologically. Patients with extraocular extension were selected based on their pathology report.

Informed consent was obtained before treatment and the study was performed according to guidelines of the Declaration of Helsinki. Until 1999, all patients were enucleated; hereafter, enucleation was performed only if the tumor was too large for fSRT (basal tumor diameter > 16 mm and tumor thickness > 12 mm) or if the patient requested enucleation.[17] MFS was defined as the time in months from enucleation until the development of metastasis. We obtained survival data up to April 2013 by reviewing patients' charts and contacting their primary physician. Patients were screened for the presence of metastasis by testing liver enzymes in peripheral blood every 6 months for the first 5 years and thereafter annually. If these were elevated, an abdominal US or computed tomography scan was carried out.

Histopathology

Fresh tumor material was obtained within 1 hour of enucleation and processed for further histopathological and cytogenetic analysis. Conventional histopathological examination with hematoxylin and eosin (H&E) staining of formalin- fixed and paraffin-embedded eyes was performed on all tumors and confirmed the origin of the tumor. The intraocular part of the tumors were evaluated for the presence of inflammation and necrosis. Inflammation was defined as any obvious clusters of lymphoid inflammatory cells in the tumor assed by H&E staining. Microfoci of necrosis were accepted as positive. H&E staining was used to differentiate between an epithelioid, mixed, or spindle-cell type according to the modified Callender classification. Extracellular matrix patterns were visualized in tumor specimens stained with periodic acid-Schiff (PAS) reagent. The mitotic rate was determined only in tumors with extraocular extension by counting the mitosis in 8 mm^2 equal to 50 high- power fields. Extraocular extension was confirmed by revision of all histopathological sections by an ophthalmic pathologist (RV), and was defined as tumor growth through the sclera and beyond the outer scleral surface. Subsequently, the largest diameter of the extension of the tumor on the scleral surface was measured. The surgical margin was examined for infiltrating UM cells extending from the extraocular extension. We determined the route of extraocular spread and involvement of optic nerve, ciliary body, or choroid.

Cytogenetic Analysis

We determined the copy number status of chromosomes 3 and 8 of the intraocular part of the primary tumor with fluorescence in situ hybridization (FISH) analysis by using centromeric and locus-specific probes on directly fixated tumor cells for chromosomes 3 and 8. A deletion was scored if more than 15% of the nuclei showed one signal for centromere 3 (probe Pα3.5) and/or 3q24 (probe YAC 827D3). Amplification was scored if more than 10% of the nuclei had three or more signals for 8q22 (probe RP-11- 88J22). For tumor samples collected from December 2000, we used a probe located on 3q25 (RP11-64F6). FISH analysis was performed in most tumors. In some tumors, the chromosome status was solely based on comparative genomic hybridization (CGH) (*n* = 8), karyotyping (*n* = 18), or single nucleotide polymorphism (SNP) array (*n* = 21). In 78 tumors, both FISH and SNP array were used to determine monosomy 3 or gain of 8q. CGH and FISH analysis were performed according to the protocol described by Naus *et al.*[18] For whole genome analysis, we used an SNP array (Illumina HumanCytoSNP-12 v2.1 BeadChip and Illumina 610Q Bead-Chip; Illumina, San Diego, CA). Two hundred nanograms of fresh tumor DNA was used as input. The data were analyzed with version 6 of the Nexus software (Biodiscovery, Inc., El Segundo, CA). BioDiscovery's SNP-Rank Segmentation Algorithm, an extension of the Rank Segmentation algorithm (a statistically based algorithm similar to the Circular Binary Segmentation algorithm[19]), was used to make copy number as well as loss of heterozygosity (LOH) calls. SNP-Rank Segmentation takes into account both the log-R as well as the B-allele frequency value at each probe location to create a segment. The significance threshold for segmentation was set at 5.0E-7, also requiring a minimum of three probes per segment and a maximum probe spacing of 1000 kilobase pairs (Kbp) between adjacent probes before breaking a segment. The log ratio thresholds for single copy gain and single copy loss were set at 0.15 and -0.15, respectively. The log ratio thresholds for two or more copy gain and homozygous loss were set at 0.41 and -1.1, respectively. The homozygous frequency threshold was set to 0.95. The homozygous value threshold was set to 0.8. The heterozygous imbalance threshold was set to 0.4. The minimum LOH length was set at 100 Kbp. Polyploid tumors with a relative loss of chromosome 3 were also considered as monosomy 3 UM. This is also applicable for relative gain of chromosome 8q.

Statistical Analysis

Tumors with an epithelioid and mixed cell type were classified as tumors containing epithelioid cells for further statistical analysis. The primary end point for MFS was the development of metastatic disease. Cases in which the cause of death was unknown or not related to their UM, were treated as censored. The importance of prognostic factors on MFS was assessed using the log-rank test (for categorical variables) or Cox regression analysis (for continuous variables).

The significance of associations between clinico-histopathological, chromosomal variables and extraocular extension were calculated with the Pearson's χ^2 test or Fisher's exact test (for categorical variables) and the Mann-Whitney test (for continuous variables). Multivariate analysis using the forward stepwise method was conducted for the variables that were significant in univariate analysis. A two-tailed P value less than or equal to 0.05 was considered significant. Statistical analyses were performed with SPSS version 20.0 software (SPSS, Inc., Chicago, IL).

RESULTS

Patients

In total, 357 patients were included in this study. The mean age was 61 years at time of enucleation (range, 21 –90). The mean largest basal tumor diameter was 12.5 mm (range, 2.0 – 22.0) and the mean tumor thickness was 7.3 mm (range, 1.0 – 24.0). Overall, 20 patients received fSRT as initial treatment, of whom two patients had extraocular extension. Two patients received brachytherapy and one patient received proton beam radiation before enucleation. These patients did not have extraocular extension. Genetic testing of the UM patient who received proton beam radiation was conducted 20 months after the radiation and revealed a normal chromosome 3 and 8 status.

The tumor characteristics for the patients with extraocular extension versus patients without extraocular extension are shown in **Table 1**. Extraocular extension was identified in 43 (12%) of 357 patients (**Figure 1A**). The mean age of the patients with extraocular extension was 64 years (range, 29 – 86). The mean largest basal tumor diameter and mean tumor thickness for this group of patients with extraocular extension were 14.2 mm (range, 6.0 – 22.0) and 7.7 mm (range, 1.5 – 22.0), respectively. Tumor localization (P = 0.043) and largest basal tumor diameter (P = 0.002) correlated with extraocular extension (**Table 1**).

Table 1. Tumor characteristics in uveal melanoma patients with and without extraocular extension (EXE)

Variable	Correlations			Univariate survival analysis	
	Patients without EXE (*n* = 314)	Patients with EXE (*n* = 43)	P-value		P-value
Age at enucleation (*n* = 357), mean, y (range)	60.2 (21-90)	63.9 (29-86)	0.085*	HR = 1.019	**0.002**†
Gender (*n* = 357)					
Male, *n* (%)	161 (51.3)	29 (67.4)	0.051‡	164.7 mo	0.617§
Female, *n* (%)	153 (48.7)	14 (32.6)		172.5 mo	
Tumor location (*n* = 357)					
Choroid, *n* (%)	289 (92.0)	35 (81.4)	**0.043**‡	170.8 mo	0.249§
Ciliary body, *n* (%)	25 (8.0)	8 (18.6)		135.4 mo	
Tumor size					
Largest basal tumor diameter (*n* = 356), mean, mm (range)	12.3 (2.0 - 21.0)	14.2 (6.0 - 22.0)	**0.002***	HR = 1.138	**< 0.001**†
Tumor thickness (*n* = 355), mean, mm (range)	7.2 (1.0 - 24.0)	7.7 (1.5 - 22.0)	0.778*	HR = 1.060	**0.003**†
Epithelioid cells (*n* = 356)					
Absent, *n* (%)	113 (36.1)	16 (37.2)	0.868‡	188.2 mo	**0.002**§
Present, *n* (%)	200 (63.9)	27 (62.8)		154.6 mo	
Extracellular matrix patterns (*n* = 302)					
Absent, *n* (%)	148 (57.1)	19 (44.2)	0.136‡	167.6 mo	**< 0.001**§
Present, *n* (%)	111 (42.9)	24 (55.8)		87.1 mo	
Inflammation (*n* = 255)					
Absent, *n* (%)	50 (23.6)	33 (76.7)	**< 0.001**‖	162.0 mo	0.686§
Present, *n* (%)	162 (76.4)	10 (23.3)		163.0 mo	
Extraocular extension (*n* = 43)					
Largest periscleral diameter of the EXE, mean, mm (range)	0	2.9 (0.1 - 40.0)	**< 0.001***	HR = 1.120	**< 0.001**†
Loss of chromosome 3 (*n* = 286)					
Absent, *n* (%)	96 (38.9)	14 (35.9)	0.860‡	151.6 mo	**< 0.001**§
Present, *n* (%)	151 (61.1)	25 (64.1)		96.8 mo	
Gain of chromosome 8q (*n* = 279)					
Absent, *n* (%)	80 (33.2)	11 (28.9)	0.711‡	169.8 mo	**< 0.001**§
Present, *n* (%)	161 (66.8)	27 (71.1)		97.1 mo	

Abbreviations: EXE = extraocular extension; y = years; HR = hazard ratio; mo = months;

* = Mann-Whitney test; † = Cox regression analysis; ‡ = Fisher's exact test; § = Log-rank test; ‖ = χ^2-test

The P-values that were significant (defined as P-value less than or equal to 0.05) are shown in bold

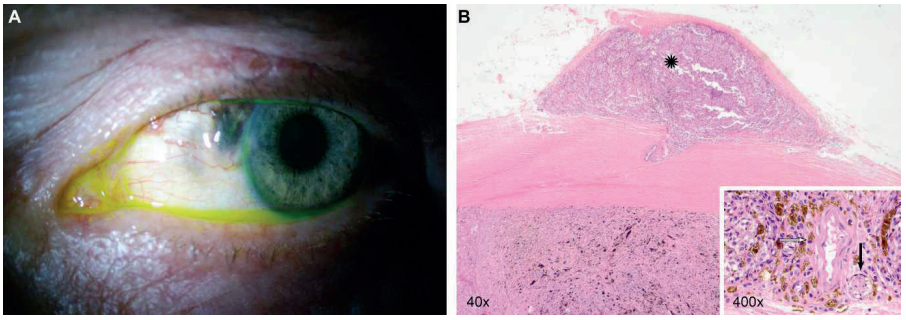

Figure 1. Slit lamp photograph of a ciliary body melanoma with extraocular extension (**A**). Extraocular extension of choroidal melanoma (indicated with *asterisk*) in hematoxylin and eosin (H&E) staining (40x) and an enlargement is shown with perineural (*black arrow*) and perivascular invasion (*white arrow*) of extraocular extension in H&E staining (400x) (**B**).

Histopathology

Several histopathological features were determined for the extraocular extension (**Figure 1B**). For instance, the (largest) episcleral diameter of the extraocular extension ranged from 0.1 to 40.0 mm, with a mean of 2.9 mm. Necrosis was found in 23 of 43 histopathological slides. The mean mitotic rate was 9.95/8 mm^2 (range, 0.00 – 29.00). Absence of inflammation ($P < 0.001$) was associated with extraocular spread (**Table 1**). Eleven of the choroidal tumors invaded the ciliary body and all the ciliary body tumors invaded the choroid. The tumors did not show a significant difference in size of the mean largest basal tumor diameter and mean tumor thickness within the extraocular extension group ($P = 0.615$ and $P = 0.517$, respectively)). Combinations of several routes of invasion of the extraocular extension were observed (**Figure 1B**). Seven UMs invaded the perilimbal plexus, the anterior part of the eye. Five tumors invaded the equator of the eye through vortex veins and two UMs invaded through the anterior ciliary arteries. Most of the tumors with extraocular extension were located posteriorly; 11 UMs invaded through the long posterior ciliary nerve, 14 through the short posterior ciliary nerve, and 1 through both ciliary nerves. Besides these routes of invasion, 41 tumors also invaded perivascularly and 29 tumors invaded perineurally. In total, three UMs with extraocular spread invaded the lamina cribrosa through three different routes: transscleral, and short and long ciliary nerves. However, the optic nerve resection margin was free of malignant cells.

In 20 patients, infiltrating UM cells extending from the extraocular extension were observed at the surgical margin. Of these patients, seven received postoperative irradiation and one patient with a 40-mm extraocular extension underwent an orbital exenteration. Thus far, orbital recurrence was noticed in one patient with

a free surgical margin. Seven of the 20 patients with irradical enucleation were still alive at the last follow-up date. There was no significant difference in mean survival between patients with (72.7 months, 95% confidence interval (CI) 46.9 – 98.5, log-rank test, P = 0.660) and without (120.6 months, 95% CI 67.7 – 173.6) a free surgical margin.

Cytogenetic Analysis

Loss of chromosome 3 was present in 61.5% (176/286) of all UMs and in 64.1% (25/39) of the tumors with extraocular extension (**Table 1**). This was not statistically different from cases without extraocular extension. Gain of chromosome 8q was present in 67.4% (188/279) of all UMs and in 71.1% (27/38) of the UMs with extraocular extension. Forty-seven patients had gain of 8q with disomy of chromosome 3, and 141 patients had gain of 8q combined with monosomy 3. An example of a case with loss of chromosome 3 and gain of chromosome 8q on SNP array is depicted in **Figure 2**. Twenty- two extraocular extension patients showed gain of chromosome 8q combined with monosomy 3. Due to lack of material, chromosome 3 and 8 status could not be examined in all extraocular extension patients.

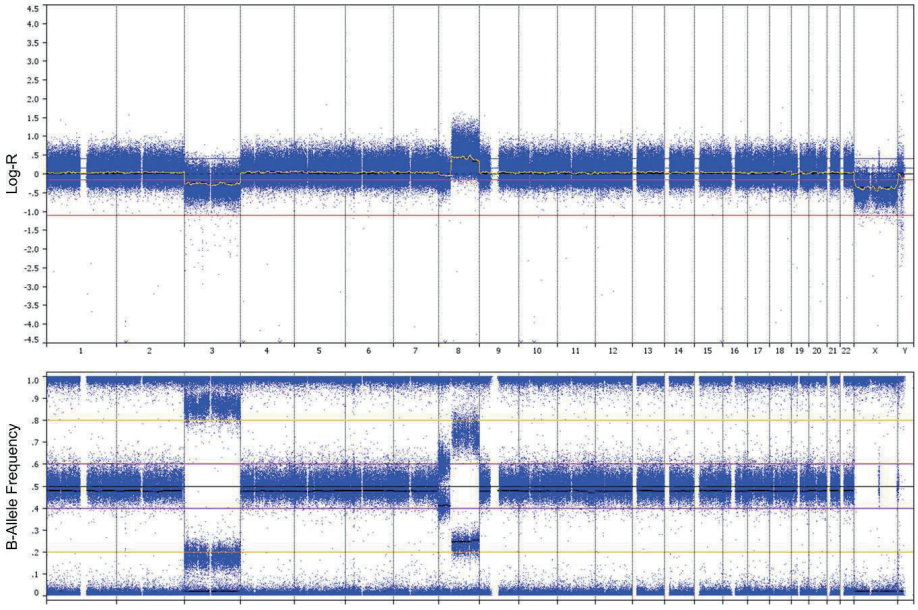

Figure 2. Single nucleotide polymorphism (SNP) array results of a male patient with uveal melanoma, showing loss of chromosome 3 and gain of chromosome 8q (Log-R, *top*). The *bottom* represents the B-allele frequency showing loss of heterozygosity (LOH) and allelic imbalance of chromosome 3 and 8, respectively.

Survival Analysis

The mean MFS of the overall group was 76.6 months (range, 0.0–308.5). Irrespective of extraocular extension, 145 patients (40.6%) developed metastasis with a mean survival of 41.8 months (range, 0.0–207.7) and 158 patients (44.3%) were alive at the end of the follow-up with a mean survival of 104.3 months (range, 0.8–308.5). Forty-three patients (12.0%) died due to other disease causes, such as a ruptured aneurysm or a myocardial infarction. The mean survival of this group was 93.4 months (range, 0.2–270.0). Eleven patients were lost to follow-up, of which five patients moved abroad and the other six patients moved to another city and did not provide their general practitioner with information or withdrew from ophthalmologic follow-up.

The follow-up of patients with extraocular extension is shown in **Table 2**. The survival was significantly reduced in patients with extraocular extension versus without extraocular extension (105.5 vs. 175.8 months, respectively, log-rank test, P = 0.010).

Table 2. Follow-up of patients stratified for the presence of extra-ocular extension (EXE)

	Patients without EXE (*n* = 314), *n* (%)	**Patients with EXE (*n* = 43), *n* (%)**
Alive	144 (45.9)	14 (32.6)
Melanoma-related death and metastases	121 (38.5)	24 (55.8)
Death due to other cause	38 (12.1)	5 (11.6)
Lost to follow up	11 (3.5)	0 (0.0)

Abbreviations: EXE = extraocular extension

Univariate analyses of prognostic factors showed a significantly shorter MFS in tumors with a larger episcleral diameter of the extraocular extension (hazard ratio (HR) 1.120, P < 0.001), epithelioid cells (154.6 vs. 188.2 months, P = 0.002), extracellular matrix patterns (87.1 vs. 167.6 months, P < 0.001), monosomy 3 (96.8 vs. 151.6 months, P < 0.001), and gain of 8q (97.1 vs. 169.8 months, P < 0.001) (**Table 1**). In addition, we conducted univariate survival analysis for extraocular extension UM patients only and the episcleral diameter of the extraocular extension remained significant (HR 1.079, P = 0.040).

The MFS was significantly longer in the overall group without chromosomal aberrations compared with patients with these aberrations (**Figures 3A** and **3C**). UM with disomy 3 and normal 8q versus gain of 8q (171.6 vs. 123.2 months, P = 0.004) showed a prolonged survival compared with UM with monosomy 3 and normal 8q versus gain of 8q (143.5 vs. 78.1 months, P < 0.001).

In the subgroup of extraocular extension, patients with and without monosomy 3 had a survival of 73.5 months and 92.0 months, respectively (P = 0.056) (**Figure 3B**). Patients with extraocular extension and gain of 8q had a reduced survival compared with patients with normal chromosome 8q (P < 0.001) (**Figure 3D**). We validated the interaction between extraocular extension and gain of 8q and its effect on the MFS in a separate multivariate model.

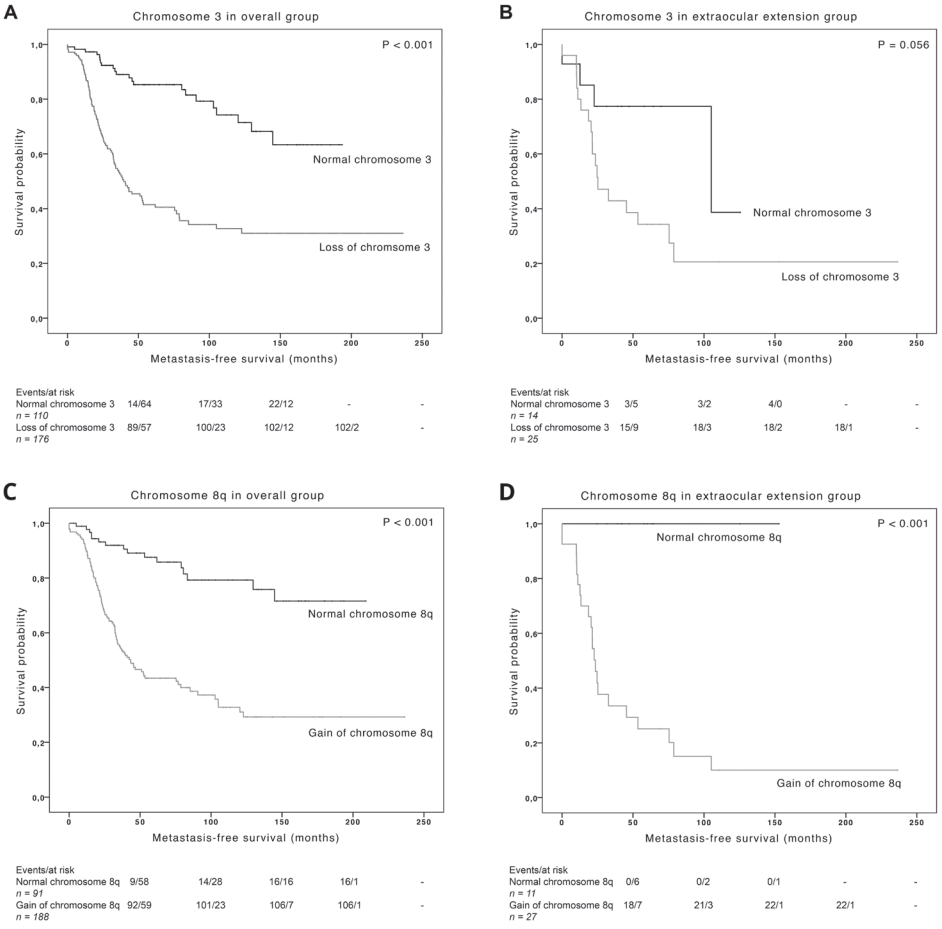

Figure 3. Survival probability plots for chromosomes 3 and 8q in the overall group (**A, C**) and in the extraocular extension group (**B, D**).

In multivariate analysis, the largest basal tumor diameter, extracellular matrix patterns, episcleral diameter of the extraocular extension, chromosome 3 loss (HR 2.634, P < 0.001), and chromosome 8q gain (HR 2.874, P < 0.001) were independent prognostic factors on MFS (**Table 3**). Prognostic factors, such as presence of epithelioid cells, extraocular spread in general, tumor thickness, and age, were rejected after multivariate analysis.

Table 3. Cox multivariate regression analysis correlating with metastatic disease

	P-Value	HR	95% CI
Largest basal tumor diameter	0.001	1.094	1.037 – 1.155
Extracellular matrix patterns	0.009	1.674	1.137 – 2.464
Largest episcleral diameter of the extraocular extension	0.016	1.078	1.014 – 1.147
Loss of chromosome 3	< 0.001	2.634	1.570 – 4.420
Gain of chromosome 8q	< 0.001	2.874	1.651 – 5.005

Abbreviations: HR = hazard ratio; CI = confidence interval

DISCUSSION

UM patients with extraocular extension are a clinically challenging group of patients, as there are only a few studies that have a large cohort of patients for analysis and often a limited duration of follow-up. In our study, we reviewed 357 ciliary body and choroidal UMs, of which 43 (12%) had extraocular extension with a mean follow-up of 6.4 years (range, 0.0 – 25.7 years). As observed in previous studies, we also found that UM patients with loss of chromosome 3 and/or gain of chromosome 8q in their melanoma have a significantly reduced MFS (P < 0.001). In addition, we observed that gain of chromosome 8q was associated with a worse prognosis in patients with extraocular extension. Besides that, patients with extraocular extension developed metastases or died due to metastases almost 6 years earlier, on average, compared with patients without extraocular extension (log-rank test, P = 0.01).

Monosomy 3 and gain of chromosome 8q (or concurrent presence of abnormalities on chromosomes 3 and 8) and extraocular extension have already separately been identified as risk factors in several other studies.[3,11,13,14,20] Nevertheless, gain of chromosome 8q in combination with extraocular extension has not been related to survival. A near significant trend (P = 0.056) was observed between monosomy 3 and extraocular extension regarding survival. With a larger patient group, a relation to patient survival could be noted. Histopathological factors have been described and related to survival in patients with extraocular extension. Coupland and associates[5] found that epithelioid cell type and high mitotic rate

were related to extraocular spread and poor prognosis. In our series, UMs with an epithelioid cell type were also related to a reduced survival, though this did not correlate with extraocular extension (P = 0.868). Because the percentages of epithelioid cells in the group of extraocular extension and without extension were similar, and although a difference in survival was measured, epithelioid cell type appeared not to be the most important prognostic factor in our population. In our multivariate analysis, the presence of epithelioid cells, extraocular spread in general, tumor thickness, and age were rejected. These prognostic factors were significant predictors of survival in the univariate analysis. In the multivariate analysis, age nearly reached statistical significance as an independent prognostic marker (P = 0.051). In previous studies, older age and presence of epithelioid cells have proven to have a significant effect on survival.[3,5]

In concordance with previous studies, we also found that clinical factors, such as a larger basal tumor diameter and the presence of extracellular matrix patterns, were associated with a decreased survival, whereas the size of the extraocular extension did not correlate significantly with metastatic death in all studies.[3,5,14] With an increasing size of extraocular extension diameter, the 5-year survival seems to decline: 81% in patients without extension, 49% in patients with a 0.1- to 5.0-mm extension diameter, and 18% in patients with 5.1-mm or more extension diameter.[14] In our analysis, we found that an increase of 1 mm in episcleral diameter led to a nearly 1.1 times increase in risk of developing metastatic disease (HR 1.078). We had only three patients in the subcategory of greater than or equal to 5.1 mm, and for this reason we could not perform statistical analyses for this group. In these three patients, one patient had metastasis at time of diagnosis (diameter extension of 40 mm) and another patient died due to a non–melanoma-related cause without metastasis (diameter extension of 9 mm) with a follow-up of 110.4 months at an age of 86 years. The third patient with a 6-mm extension was still alive at 41.9 months and had other favorable prognostic factors, such as the absence of genetic aberrations, absence of extracellular matrix patterns, absence of mitotic figures, and a free surgical margin. Interestingly, all three UMs contained epithelioid cells.

Orbital recurrence has been reported in 3% to 23% of the patients undergoing enucleation for UM with extraocular extension.[16,21-23] In our study, only one patient, with an initial tumor-free surgical margin, had an orbital recurrence after 7 months and was exenterated. Nevertheless, orbital recurrence is described even 20 and 42 years after enucleation.[24] In 20 of 43 UM patients with extraocular spread, melanoma cells extending from the extraocular extension were found at the surgical margin. Of the irradiated patients, the mean survival was 71.2 months (range, 10.4 – 257.1), and was almost similar to patients without additional treatment, 79.2 months (range, 0.0 – 254.2). Nevertheless, incomplete surgical removal of the tumor, especially if the extraocular part of the tumor

is nonencapsulated, remains one of the most important risk factors for orbital recurrences.[23] In our group of patients with incomplete resection, we found no cases with orbital recurrence. Nowadays most patients with extraocular extension will be treated with additional therapy or surgery.

In this study, we associated extraocular extension with chromosomal abnormalities of chromosomes 3 and 8 in UM. Compared with other studies, our patient group has a long follow-up with a mean MFS of 6.4 years, and only a few patients were lost to follow-up. Extraocular extension was histologically proven and reviewed by an ocular pathologist in a relatively large group of UMs. Because this is a retrospective study, some data were missing. For example, we could not detect histopathological or chromosomal aberrations in all patients due to necrosis or lack of material. Because we studied only enucleated eyes and not patients who have had eye-conserving treatments, our group contained relatively large UMs. This selection bias could influence survival, because in general larger tumors have a worse prognosis. Still, in our multivariate analysis, other parameters remained significantly associated with a decreased survival. Chromosome 3 and 8q status was determined in almost all patients with FISH, and in some cases with additional SNP array analysis. Intratumor heterogeneity has been described in a small number of UMs in our research group previously, although no structural difference in monosomy 3 distribution occurred between the base and the apex of the tumor.[25] On the other hand, genetic heterogeneity of chromosomes 3 and 8 has been reported between the intraocular and extraocular part of the UM, and for monosomy 3 between the apex and base of the tumor.[26,27] This variation of monosomy 3 in intra- and extraocular parts of UM was demonstrated by Lake *et al*.[26] with multiplex ligation-dependent probe amplification (MLPA) in only 10 patients. Despite a certain heterogeneity, tumors can be classified correctly for monosomy 3 or gain of chromosomal region 8q, as is the case in our study, as we used either FISH and/or confirmed these results with SNP array in a large group of our patients. Moreover, from previous studies we know that the percentage of chromosomal aberrations does not influence the development of metastases, but can influence the time to development of metastatic disease.[12] In our series, we found that MFS is significantly reduced in UMs with a large basal tumor diameter, extracellular matrix patterns, loss of chromosome 3, and gain of chromosome 8q. Loss of chromosome 3 itself is not related to extraocular extension, but a gain of chromosomal region 8q in tumors with extraocular extension increases the risk of metastatic disease.

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CHAPTER 3

ABSENCE OF INTRAOCULAR LYMPHATIC VESSELS IN UVEAL MELANOMAS WITH EXTRASCLERAL GROWTH

Jackelien van Beek
Quincy van den Bosch
Nicole Naus
Dion Paridaens
Annelies de Klein
Emine Kiliç
Robert Verdijk

on behalf of the Rotterdam Ocular Melanoma Studygroup (ROMS)

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ABSTRACT

The aim of this study was to investigate the presence of intraocular lymphatic vessels in patients with uveal melanomas and extrascleral extension using a panel of lymphatic markers. The following immunohistochemical markers were analyzed: lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1), podoplanin (D2-40), prospero-related homeobox gene-1 (Prox-1), pan-endothelial marker cluster of differentiation 31 (CD31), and blood vessel endothelium-specific CD34. Lymphatic vessels were defined as a combination of staining of the following positive markers: LYVE-1, D2-40, Prox-1, and CD31; and no staining of the negative marker CD34. In total, 456 patients were enucleated; 16 of the 46 uveal melanomas with extrascleral extension were contained in stored paraffin tissue. Two samples of the 16 uveal melanomas showed focal positive intraocular vascular staining for LYVE-1 and co-expression of CD31 and CD34. Due to the lack of Prox-1 and D2-40, and positive expression of CD34, these cannot be classified as lymphatic vessels. In one case recruitment of an extraocular, intratumoral lymphatic vascular structure was observed in the periphery of the subconjunctival extrascleral extension. Intraocular lymphatic vessels are absent in uveal melanomas with extrascleral extension; however, we provide proof for recruitment of intratumoral lymphatics by uveal melanomas with extraocular extension from subconjunctival lymphatics that may explain the rare cases of regional lymphatic spread. A panel of antibodies is necessary to detect lymphatic vessels with high specificity.

INTRODUCTION

Ocular melanoma comprises 3% of all melanomas and may originate intraocularly from the uvea or extraocular from the conjunctiva.[1] In contrast to conjunctival melanoma, uveal melanomas (UM) are known to metastasize primarily in a hematogenous manner. Lymphatic metastasis of UM in regional lymph nodes is exceptionally rare and is associated with extraocular extension or orbital recurrence.[2-5] Extraocular extension occurs in 2-15% of UM and is associated with decreased survival.[6] Various routes for the extraocular extension were described, such as aqueous channels, ciliary arteries, vortex veins, ciliary nerves, optic nerve, and a variety of rare combinations of these routes.[7] Extraocular spread of UM may provide access to subconjunctival lymphatics and, moreover, was hypothesized by some to promote intraocular lymphangiogenesis through recruitment.[5] The lymphatic system is important in providing the products for immune response, interstitial fluid balance, and macromolecular absorption (lipids). The vessel wall consists of the tunica intima, media, and adventitia; however, it is less organized than in blood vessels. There is functional evidence for lymphatic-like tissue in the anterior uvea, and true lymphatic vessels are present in the bulbar conjunctiva.[8] The choroid is known to be alymphatic and whether or not intraocular "classical" lymphatics exist is, therefore, controversial. Several studies suggested that lymphatic vessels are present in the human eye and are supposedly involved in lymphatic metastasis of intraocular malignancies. Lymphatic vessels are derived from venous endothelial cells during embryogenesis. Fetal human sclera is free of lymphatic vessels, but not of blood vessels.[9] Some reported pseudo-lymph vessel appearance in human choroid, while others proposed that intraocular lymphatic vessels with continuous endothelial lining do not exist.[8,10] Tumor-associated ocular lymphangiogenesis was detected in the most common malignant ocular surface tumors of the eye, such as conjunctival carcinoma and melanoma.[11-13] Peritumoral intraocular lymphatic vessels were also reported in UM with and without extraocular extension, and it was suggested that intraocular lymphangiogenesis in ciliary body melanomas with extraocular extension may be considered as a new prognostic factor.[5,14,15] However, this depends on which definition is used for a lymphatic vessel.

Lymphatic vessels can be identified using a panel of immunohistochemical markers.[16-18] Lymphatic endothelial markers are expressed in lymphatic endothelium, but not in blood vessel endothelium. New endothelial markers recognize growth factors and differentiation antigens specific for lymphatic endothelial cells. It is recommended to use a minimum panel of three endothelial antibodies, which consists of one pan-endothelial marker and at least two different lymphatic endothelial-specific antibodies, to avoid overinterpretation of false staining results.[16] Podoplanin is a mucin-type transmembrane glycoprotein, expressed in lymphatic endothelial cells and other cells such as macrophages and

tumor cells.[19,20] Its function includes regulation of lymphatic vascular formation and platelet aggregation. D2-40 is the most commonly used mouse monoclonal antibody against podoplanin. Prospero-related homeobox gene-1 (Prox-1) is an important lymphatic differentiation factor, a nuclear transcription factor, and of importance for the development of the lymphatic system. Prox-1 is also expressed in nonendothelial cell types, such as hepatocytes, bile duct epithelium, pancreatic epithelium, cardiomyocytes, lens, retina, and spinal and vegetative ganglia.[21,22] It is not expressed in blood vascular endothelial cells, except for a small segment of the embryonic anterior cardinal vein.[23] Knockout models of Prox-1 discovered the crucial role of Prox-1 in the development of the lymphatic system.[24] Lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) is an integral membrane glycoprotein and lymphatic vessel endothelial hyaluronan receptor type 1. LYVE-1 is expressed in lymphatic, but not in blood vascular endothelium.[19] LYVE-1 may also be expressed by activated macrophages.[25] Cluster of differentiation 31 (CD31; platelet endothelial cell adhesion molecule-1, PECAM) is the most sensitive and specific pan-endothelial marker. It is an integral membrane glycoprotein, expressed on endothelial intercellular junctions, but may also be expressed by macrophages.[26] The marker CD34 is a single-chain transmembrane glycoprotein, a hematopoietic progenitor cell antigen, and is expressed in the endothelial cells of blood vessels, but not in non-neoplastic lymphatic vessels. All these markers may also be expressed in other cells than lymphatic endothelium; therefore, it is difficult to identify lymphatic vessel based on a single marker. When multiple markers are used, lymphatic vessels will be identified with increased accuracy.

Earlier published data suggested the presence of intraocular lymphatic vessels in UM with and without extrascleral extension; however, this was based on restricted immunohistochemical panels.[5,14] Therefore, we analyzed the presence of lymphatic vessels in eyes with UM and extrascleral extension with an extensive panel of lymphatic markers. It is important to interpret the staining pattern with care in order to identify a vascular structure as a lymphatic vessel.[16] In the current study, a lymphatic vessel was identified when it showed expression of the following markers: D2-40, Prox-1, LYVE-1, and CD31, and lacked expression of CD34.

METHODS

Sample Selection

Eyes of patients with a choroidal or ciliary body UM with extraocular extension, who underwent primary or secondary enucleation, were selected from the Rotterdam Ocular Melanoma Studygroup (ROMS) database from 1993 until 2016.[6] Sample size was not determined statistically in advance. Patients with iris melanoma and

patients without a pathology report, describing the histopathological characteristics of the tumor, were excluded. Tumor characteristics, the largest tumor diameter and the tumor thickness, were measured on B-scan ultrasonography and, if those results were unknown, we used the measurements from the pathology report. This was also done for the diameter of the extraocular extension. Until 1999, all patients were enucleated; hereafter, enucleation was only performed if the tumor was too large for fractionated stereotactic radiotherapy (basal largest tumor diameter >16 mm and tumor thickness >12 mm) or if the patient requested enucleation. Disease-free survival was defined as the moment of diagnosis until development of metastases or patient death. Cases in which the cause of death was unknown or not related to UM were treated as censored. Survival data was obtained until July 2017. The oncologist determined if there were metastases, and patients received routine blood tests during follow-up or ultrasound of the liver. Informed consent was obtained prior to treatment, and the study was performed according to guidelines of the Declaration of Helsinki.[27] The Medical Ethics Committee of the Erasmus MC approved this study (MEC-2009-375).

Tissue Processing

Fresh tumor material was obtained within one hour of enucleation, and the globes were processed for further histopathological analysis. Conventional histopathologic examination with hematoxylin and eosin (H&E) staining of formalin-fixed and paraffin-embedded (FFPE) eyes was performed on all tumors and confirmed the origin of the tumor, presence of inflammation, and necrosis. H&E staining was used to differentiate between an epithelioid, mixed, or spindle cell type according to the modified Callender classification.[28] Extraocular extension was defined as tumor growth through the sclera and beyond the outer scleral surface. Subsequently, the largest diameter of the extension of the tumor on the sclera surface was measured. The orbital fat resection margin was examined for presence of malignant cells. We determined the route of extraocular spread and involvement of optic nerve, ciliary body, or choroid.

Immunohistochemistry

FFPE tumor tissue of uveal melanomas with extrascleral extension of 46 patients was analyzed for the presence of lymphatic vessels. Four-micrometer-thick sections were stained for podoplanin (Clone D2-40, reference no. 760-4395, Cell Marque, Rocklin, California, USA), prospero homeobox-1 (Prox-1, Clone D2J6J, dilution 1:1500, Cell signaling, Leiden, The Netherlands), cluster of differentiation 31 (CD31, Clone JC70, reference no. 760-4378, Cell Marque, Rocklin, California, USA), and cluster of differentiation 34 (CD34, Clone QBEnd/10, reference no. 790-2927, Ventana, Tucson, AZ, USA) with the Ventana Benchmark Ultra automated

staining system (Ventana Medical Systems, Tucson, AZ, USA). Briefly, after deparaffinization, the sections were processed for 32–64-minute antigen retrieval using Cell Conditioning Solution 1 (CC1 Ventana reference no. 950-124). Following 32-minute incubation (16-minute for CD31) with the primary antibody at 36 °C, detection was performed using the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana reference no. 760-501) in combination with the Amplification Kit (Ventana Ref.: 760-080). Sections were counterstained with hematoxylin II (Ventana reference no. 790-2208). For anti-LYVE-1 (Clone AF2089, dilution 1:1000, R&D Systems, Minneapolis, MN, USA), primary antibody staining was executed using the Ventana Discovery Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA). The following adaptations from the protocol were required: endogenous peroxidase was blocked using Inhibitor CM from the DISCOVERY ChromoMap DAB Kit (RUO) (Ventana, reference no.: 760-159) for four minutes. The secondary antibody incubation was performed with anti-Goat-horseradish peroxidase (HRP; Ventana reference no. 760-159) for 32 minutes. Detection was executed manually with 3-amino-9-ethylcarbazoledue (AEC) diluted in 0.2 M sodium acetate with H_2O_2. The slides were counterstained with Mayer's hematoxylin (Klinipath, Cat. 4085.9005, Duiven, The Netherlands).

Scoring of Immunohistochemistry

Three independent reviewers scored the slides: a pathologist, an ophthalmologist, and a research technician with ample experience in ophthalmic pathology. Lymphatic vessels were identified using a panel of immunohistochemical markers. For this study, a vascular structure was identified as a lymphatic vessel when it displayed an expression of podoplanin, Prox-1, LYVE-1, and CD31 and no expression of CD34. These requirements are in accordance with the first international consensus on the methodology of lymphangiogenesis quantification in solid human tumors.[16] Lymphatic vessels of the perilimbal conjunctiva served as an internal control. External control tissue was properly applied, such as lymphatic tissue of the testis. Expression of the markers in control eyes without UM was evaluated as well.

RESULTS

Patients

In total, 456 uveal melanoma patients were enucleated from 1993 until 2016, of which 417 patients underwent primary enucleation and 39 patients underwent secondary enucleation after fractionated stereotactic radiation therapy. Forty-six tumors showed extrascleral extension.[6] Following the hypothesis that expression of lymphangiogenic growth factors such as vascular endothelial growth factor-c

(VEGF-C) in UM may induce secondary lymphangiogenesis only when direct access to pre-existing lymphatic vessels is present, we selected such cases for immunohistochemical investigation.[5,29] Sixteen tumors contained extrascleral tumor extension in the stored paraffin tissue blocks and were selected for immunohistochemical investigation of tumor-associated intraocular lymphatics. For the remaining 30 tumors, tissue with visible extrascleral extension was not available. The mean largest diameter of the extension was 2.6 mm (standard deviation 2.5 mm). Patients and tumor characteristics are shown in **Table 1**. Fourteen patients underwent primary enucleation. One patient developed untreatable neovascular glaucoma and was treated with secondary enucleation. One patient was exenterated, because, at first presentation, the tumor was too large for enucleation.

Table 1. Baseline patient characteristics

Patient Characteristics	**Patients, *n* = 16**
Gender, No. (%)	
Men	7 (44)
Women	9 (56)
Age in years, mean (SD)[1]	66 (14)
Tumor size classification	
T1	3
T2	3
T3	7
T4	3
Largest diameter of the extension of the tumor in mm, mean (SD)[1]	2.6 (2.5)
Tumor location	
Choroid	9
Ciliary body	7
Cell type	
Epithelioid	4
Mixed	7
Spindle	5
Disease-free survival in months, mean (SD)[1]	77 (64)
Alive, *n*	9
Metastases, *n*	4
Death, because of uveal melanoma, *n*	3
Death other cause, *n*	3
Lost to follow-up, *n*	1

[1]SD = standard deviation

Immunohistochemistry

Immunohistochemical analysis showed intraocular peritumoral and intratumoral positive staining for one lymphatic marker in two samples (sample 8 and 15 in **Table 2**; **Figures 1** and **2**). However, these vascular structures showed co-expression of CD31 and CD34, and only focal expression of LYVE-1. Due to the lack of Prox-1 and D2-40 expression, these vascular structures cannot be classified as lymphatic vessels. Specifically, we did not find one sample that had an intraocular vascular structure positive for D2-40, Prox-1, LYVE-1, and CD31, with concurrent negative staining for CD34, as in the conjunctival control (Appendix, **Figure A3**).

Table 2. Required expression profile of lymphatic vessels and patient samples. CD—cluster of differentiation; D2-40—podoplanin; LYVE-1—lymphatic vessel endothelial hyaluronic acid receptor-1; Prox-1—prospero-related homeobox gene-1

Markers	Expression of CD31	Expression of D2-40	Expression of LYVE-1	Expression of Prox-1	Expression of CD34
Lymphatic vessel	+	+	+	+	–
Sample 1	+	–	–	–	+
Sample 2	+	–	–	–	+
Sample 3	+	–	–	–	+
Sample 4	+	–	–	–	+
Sample 5	+	–	–	–	+
Sample 6	+	–	–	–	+
Sample 7	+	–	–	–	+
Sample 8	+	–	+	–	+
Sample 9	+	–	–	–	+
Sample 10	+	–	–	–	+
Sample 11	+	–	–	–	+
Sample 12	+	–	–	–	+
Sample 13	+	–	–	–	+
Sample 14	+	–	–	–	+
Sample 15	+	–	+	–	+
Sample 16	+	–	–	–	+

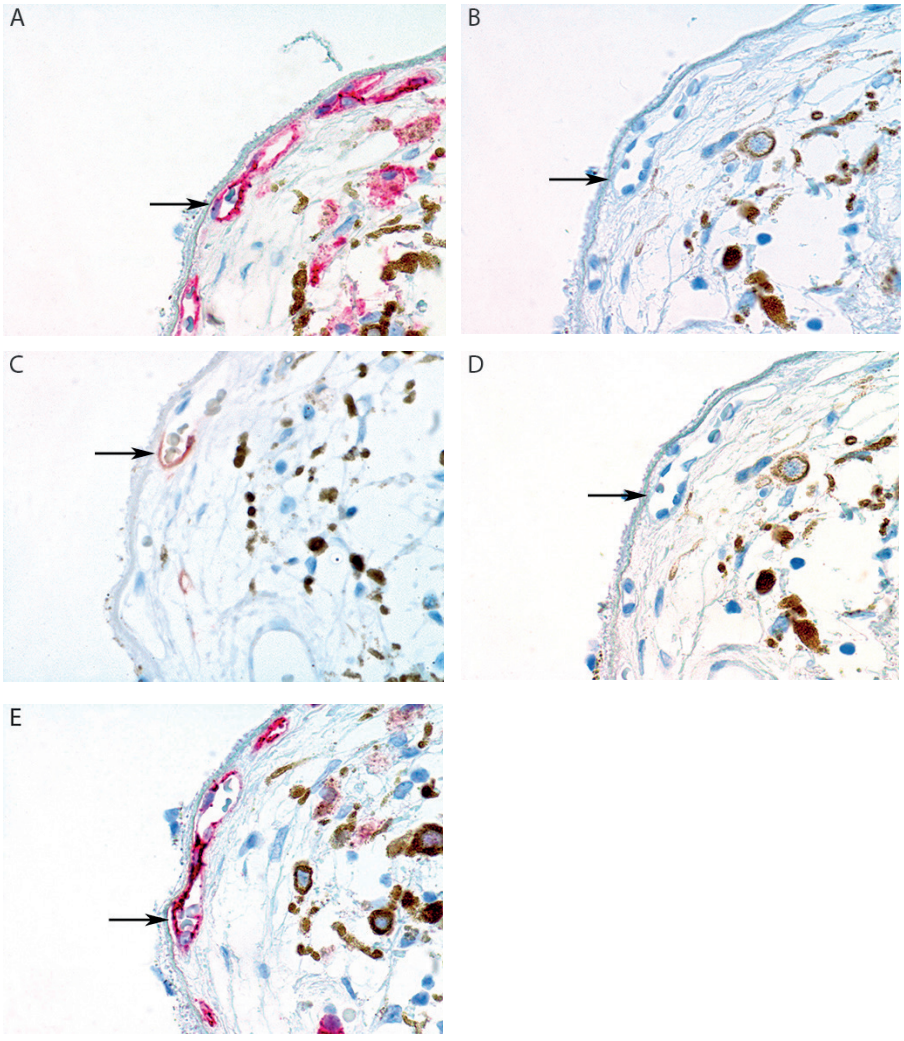

Figure 1. Sample 8 of Table 2: peritumoral focal positive staining of choriocapillary vasculature for lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1). The staining pattern of the five markers are shown (arrows). (**A**) Cluster of differentiation 31 (CD31) stains all endothelial cells. (**B**) Podoplanin (D2-40) is negative. (**C**) LYVE-1 shows focal positive staining in a vessel of the choriocapillaris. (**D**) Prospero-related homeobox gene-1 (Prox-1) is negative. (**E**) CD34 stains all endothelial cells. (All panels: original magnification 400x)

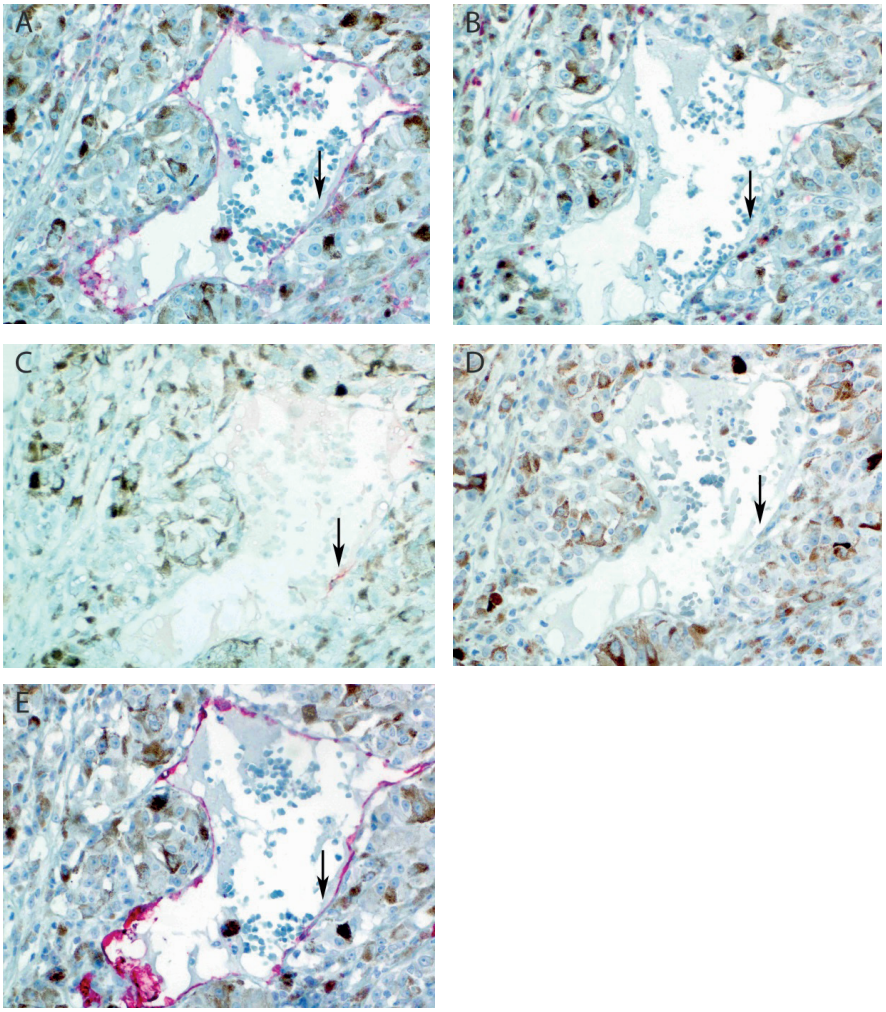

Figure 2. Sample 15 of Table 2: intratumoral focal positive staining of tumor vasculature for LYVE-1. (**A**) CD31 stains all endothelial cells (arrow). (**B**) D2-40 is negative in endothelium. (**C**) LYVE-1 shows focal positive staining in a large tumor vessel. (**D**) Prox-1 is negative. (**E**) CD34 stains all endothelial cells. (All panels: original magnification 400x)

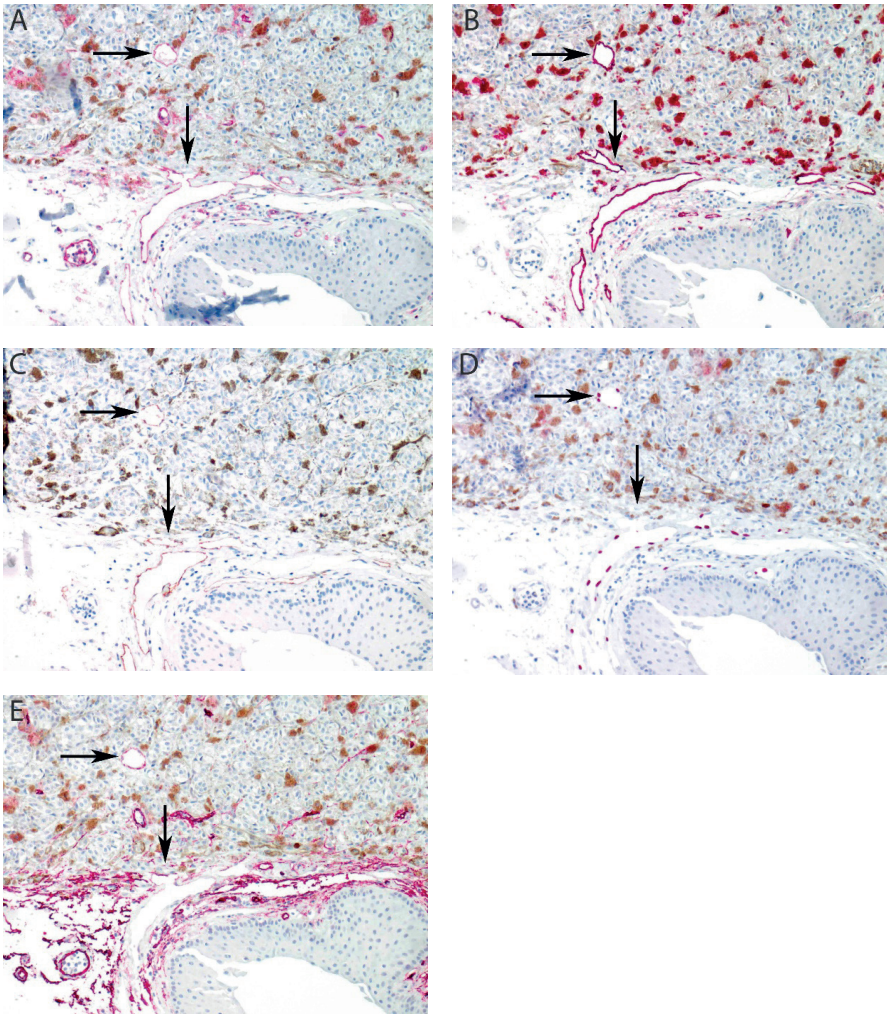

Figure 3. Recruitment of lymphatic vessels into extraocular extension of uveal melanoma (arrows). (**A**) CD31 stains all endothelial cells. (**B**) D2-40 stains conjunctival lymphatic vessel endothelium and demonstrates intratumoral recruitment. (**C**) LYVE-1 stains conjunctival lymphatic vessel endothelium and demonstrates intratumoral recruitment. (**D**) Prox-1 is positive in the nuclei of lymphatic endothelial cells and demonstrates intratumoral recruitment. (**E**) CD34 is positive in blood vessel endothelium and negative in lymphatic endothelium. Note that the intratumoral recruited lymphatic vessel stains weakly positive at the recruitment front (vertical arrow) and intatumoral (horizontal arrow). (All panels: original magnification 100x)

We paid special attention to conjunctival lymphatic vessel recruitment in cases of anterior extrascleral extension of ciliary body melanomas. In one case (in addition to the two samples mentioned earlier), without showing any of intraocular lymphatic markers, an extraocular, intratumoral lymphatic vascular structure was observed in the periphery of the extrascleral extension of the tumor. However, no intraocular recruitment was observed in this case (**Figure 3**). Positive staining for D2-40 was observed in the trabecular meshwork and anterior ciliary body of eyes without UM as reported before (Appendix, **Figure A1**), as well as in cases of ciliary body melanoma (Appendix, **Figure A2**).

DISCUSSION

This study, with an extensive panel of immunohistochemical markers, examined the presence of intraocular lymphatic vessels and lymphatic vessels adjacent to the intratumoral part of the extrascleral extension of UM. Our panel of markers made it possible to distinguish lymphatic vessels with certainty from blood vessels. We could not detect intraocular lymphatic vasculature in UM samples with extraocular extension using these five specific markers. However, we did find two samples with intraocular focal vascular positivity for LYVE-1, but negative staining for Prox-1 and D2-40. Moreover, these vascular structures were positive for CD34. These two samples had T2 and T4 tumors; both had mixed cell types and were choroid and ciliary body UM, respectively (see **Figures 1** and **2**).

Our findings do not support the findings by Heindl *et al.,*[5] who detected intraocular LYVE-1 (+) and podoplanin (+) lymphatic vessels in 12 out of 20 ciliary body melanoma with extraocular extension and associated this with worse prognosis. In the extraocular tumor component, all tumors of that study showed subconjunctival peritumoral vessels that were LYVE-1 (+) and podoplanin (+), but notably no intratumoral recruitment was observed. These authors only used lymphatic markers and did not evaluate co-expression of vascular markers such as CD31 or CD34 in these proposed lymphatic vascular structures, as would be required by the consensus statement. This implies that the functionality of these structures as lymphatic vessels remains controversial, as was also emphasized by the authors.[5] Although our findings are in concordance with Khan *et al*.,[14] who described peritumoral staining for the lymphatic marker podoplanin in UM with ciliary body involvement with or without extraocular extension, we draw a different conclusion. In that study, supposed lymphatic vessels were identified with D2-40 in combination with negative staining for the blood vessel marker CD34. This study also described possible lymphatic structures in the ciliary body in eyes without UM. The staining pattern described in this study was commented on in a letter to the editor by Heindl *et. al.*[30] who rightly argued that this staining pattern merely represented earlier described endothelial marker expression in the anterior segment of the human

eye.[8,30] We are able to support this commentary by our staining results in tumor tissue and non-tumor tissue specimens. In addition, we observed peritumoral positive staining of the ciliary body and choroidal stroma by D2-40 without co-expression of LYVE-1 or Prox-1. D2-40 is, just as LYVE-1, expressed in lymphatic endothelial cells, but also in various other cells such as macrophages and in various structures in the anterior segment of the eye. This makes it important to use more than just one immunohistochemical lymphatic vessel marker. Multiple consensus statements advise, among others, a panel of at least two lymphatic markers and one vascular marker for identification of lymphatics.[16,18]

Other studies support our observations and did not observe any intraocular lymphatic vessels in uveal melanoma. Furthermore, although other markers were used, Clarijs *et al.*[29] found, with the presence of pan-endothelial CD31, lymphatic endothelium specific Flt-4 (Fms related tyrosine kinase 4), and blood vessel endothelial marker CD34 expression, no sufficient evidence that lymphangiogenesis was induced from preexisting blood vessels in UM. Within the eye "atypical" lymphatic marker expression might exist, such as endothelial cells of Schlemm's canal that tend to show a positive expression of Prox-1.[31] On the other hand, some lymphatic vessel function was demonstrated in special vascular structures of the eye, such as Schlemm's canal.[32] However, these cells do not show all features of terminally differentiated lymphatic endothelial cells. Moreover, the human choroid is endowed with a significant number of LYVE-1 (+) macrophages.[10]

The tumor, node, metastasis (TNM) classification of UM is based on the extent of the primary tumor and on the presence of any systemic metastases, since lymph node involvement is extremely rare.[33] It is well known that UM may metastasize to the liver (and elsewhere in the body) through hematogenous spread. Only a few cases of patients with UM and extraocular spread with regional lymph node metastasis were described. In two cases with extraocular spread, regional lymph node metastases were found, and Heindl *et al.*[5] reported regional lymph node metastasis in 3/20 patients with extraocular spread.[3,5] Ardjomand *et al.*[4] described lymph node metastases from a ciliary body ring melanoma upon repeated trabeculectomy. Tojo *et al.*[2] reported 5/77 patients who developed cervical metastases associated with prior orbital recurrence and distant metastatic disease. Our study is the first to illustrate recruitment of subconjunctival lymphatics into the extraocular extension in a case of ciliary body melanoma. This finding may indeed explain regional lymphatic spread of UM by way of access of uveal melanoma cells to the subconjunctival lymphatics through (iatrogenic) extrascleral spread of the tumor. However, no regional lymphatic metastasis was documented in our patient. It must be noted that the intratumoral recruited lymphatic structure expresses uneven staining for LYVE-1, when compared to the conjunctival lymphatic vessel, and shows weak expression of CD34. This indicates the need for further functional studies of such recruited vessels.

Macrophages were suggested to stimulate neo-lymphangiogenesis in settings of inflammation. Bone-marrow-derived CD11b+ macrophages expressed lymphatic endothelial markers such as LYVE-1 and Prox-1 under inflamed conditions in the corneal stroma of mice. In vitro experiments demonstrated that CD11b+ macrophages alone were capable of forming tube-like structures that expressed markers of lymphatic endothelium such as LYVE-1 and podoplanin.[34] Uveal melanoma may show increased numbers of CD11b+ macrophages.[35] CD31, D2-40, and LYVE-1 were found to be positive in tumor-associated macrophages. In addition, vessels in a UM or near a UM could be focally positive for LYVE-1. However, when all other lymphatic markers are negative and CD34 is positive, these structures cannot be classified as functional lymphatic vessels. LYVE-1 may be positive in non-endothelial cells, as mentioned earlier. Therefore, the earlier described positive staining could easily be seen as positive staining of the macrophages, which are normally present in the choroid. When less than these five markers are used, false positive interpretation of the staining results could be passed for proof of lymphatic vessels. This is the reason why we prefer to use more markers than already advised. Functional studies of such aberrant staining vascular structures are needed to further substantiate our point.

CONCLUSIONS

We were not able to confirm intraocular lymphangiogenesis in UM, but we do provide support to the possibility of regional lymphatic spread of UM with extraocular extension through subconjunctival lymphatics. We propose a panel of antibodies (LYVE-1, Prox-1, D2-40, CD34, and CD31) to detect intraocular lymphatic vessels with high specificity. Only when the antibodies LYVE-1, Prox-1, D2-40, and CD31 show positive staining combined with negative expression of CD34 can a vascular structure be classified as a lymphatic vessel.

ACKNOWLEDGEMENTS

We wish to acknowledge the work of Mrs. Rowen Schoonderwoerd and Dr. Hans Stoop who made important contributions to the development of the immunohistochemical staining in this study. Mr. Frank van de Panne prepared the figures.

APPENDIX

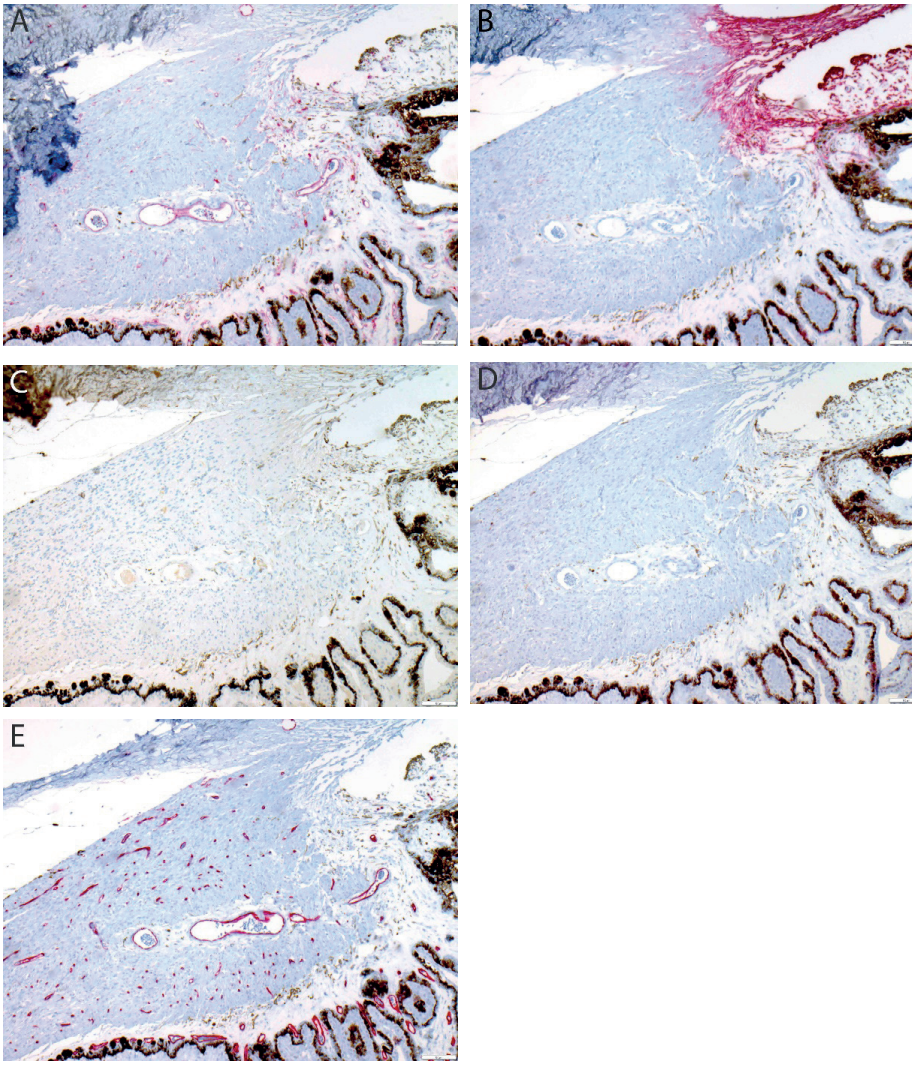

Figure A1. Positive staining of the trabecular meshwork, iris, and anterior ciliary body for podoplanin (D2-40) in an eye without uveal melanoma. (**A**) CD31 stains all vascular endothelial cells and shows focal positivity in macrophages. (**B**) D2-40 stains the stroma of the trabecular mesh work, iris, and anterior ciliary body and shows focal positivity in macrophages. (**C**) LYVE-1 is negative. (**D**) Prox-1 is negative. (**E**) CD34 stains all vascular endothelial cells. (All panels: original magnification 25x)

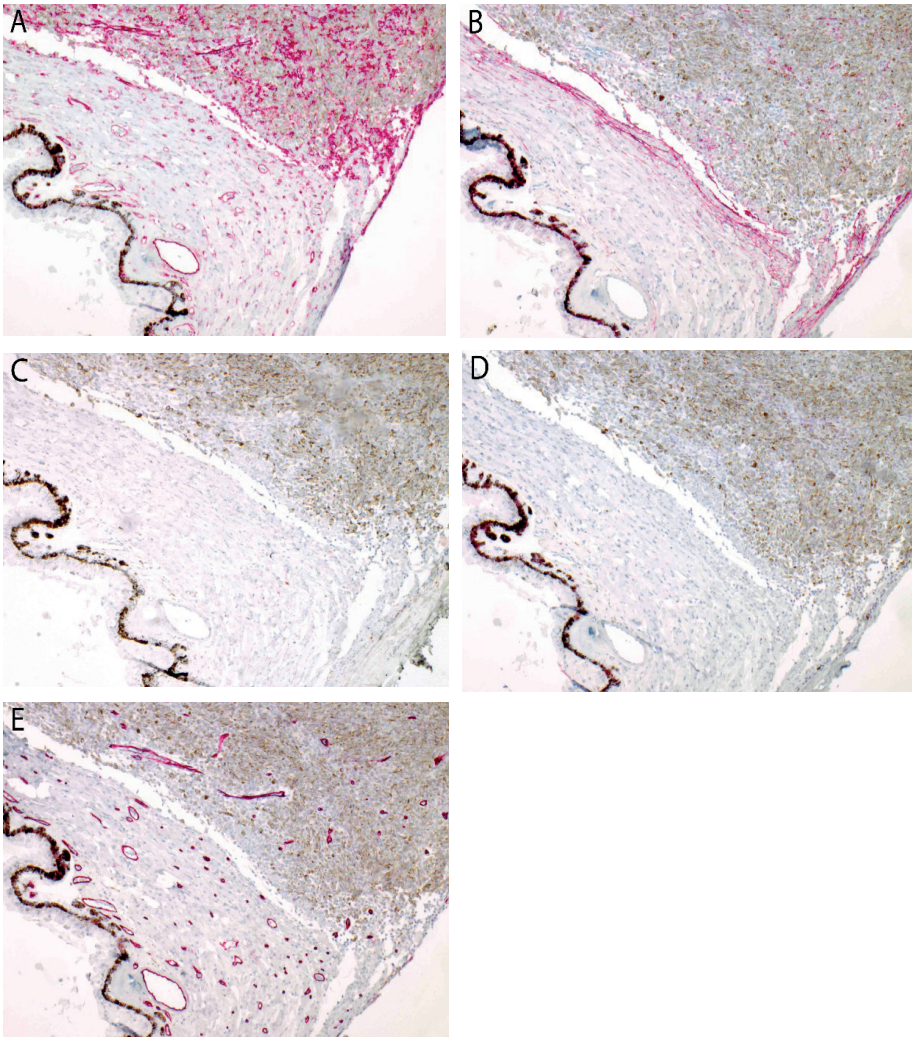

Figure A2. Positive staining of the anterior ciliary body for D2-40 in uveal melanoma. (**A**) CD31 stains all vascular endothelial cells. (**B**) D2-40 stains the peritumoral stroma of the anterior ciliary body. (**C**) LYVE-1 is negative. (**D**) Prox-1 is negative. (**E**) CD34 stains all vascular endothelial cells. (All panels: original magnification 50x)

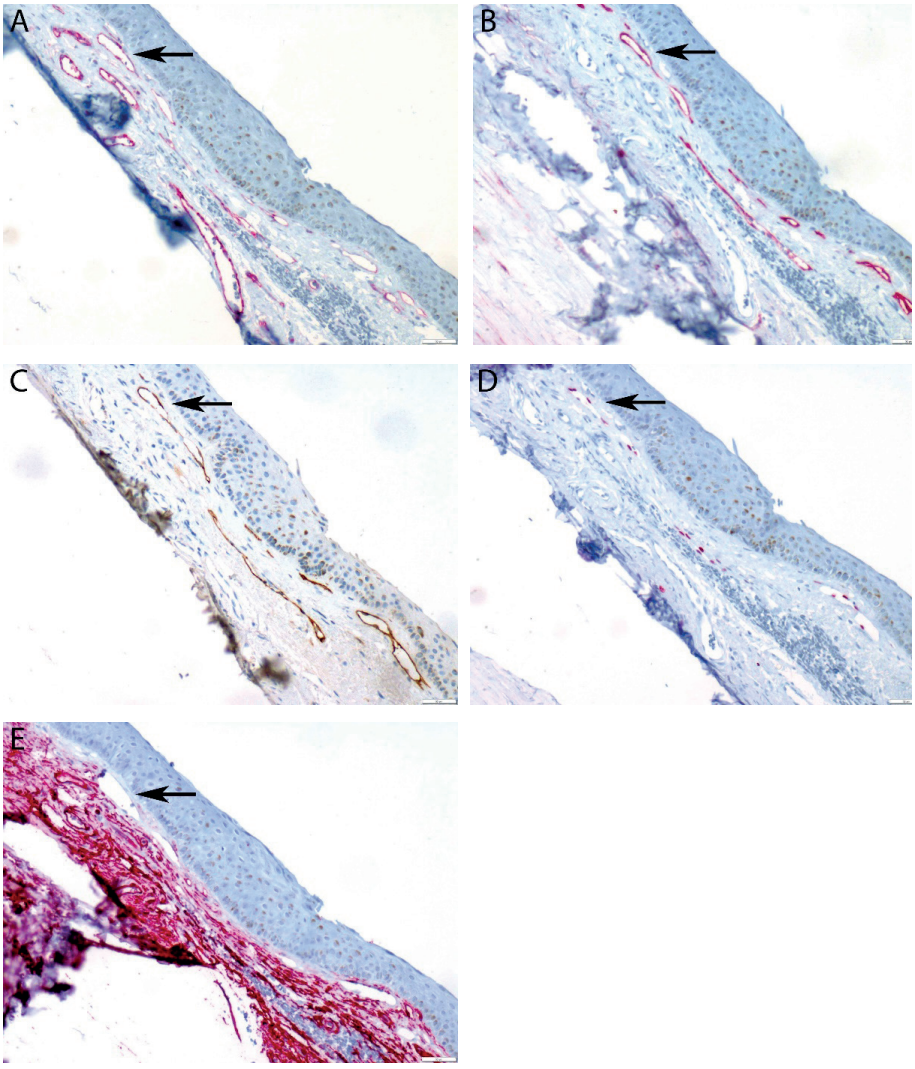

Figure A3. Conjunctival control tissue staining pattern. (**A**) CD31 stains all endothelial cells (arrow). (**B**) D2-40 stains conjunctival lymphatic vessel endothelium (arrow). (**C**) LYVE-1 stains conjunctival lymphatic vessel endothelium (arrow). (**D**) Prox-1 is positive in the nuclei of lymphatic endothelial cells (arrow). (**E**) CD34 is positive in blood vessel endothelium and negative in lymphatic endothelium (arrow). Conjunctival stroma also stains positive. (All panels: original magnification 100x)

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CHAPTER 4

TRANSIENT EXPRESSION OF LYMPHATIC MARKERS IN RETROBULBAR INTRACONAL ORBITAL VASCULATURE DURING FETAL DEVELOPMENT

Quincy van den Bosch
Jackelien van Beek
Emine Kiliç
Robert Verdijk

Invest Opthalmol Vis Sci. 2020;61(6):22

ABSTRACT

Purpose. The aim of this study is to investigate the presence of orbital lymphatic vessels during fetal and neonatal development and in adults using a panel of lymphatic markers.

Methods. This was a retrospective observational case series. For analyzing lymphatic vessels, we used formalin-fixed paraffin-embedded enucleated eyes from 25 human fetuses between 13 and 24 weeks of gestation and postnatal eyes from 15 children and 5 adults. Immunohistochemical analysis of lymphatic vessels was performed for the markers: lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1), Podoplanin (D2-40), Prospero-related homeobox gene-1 (Prox-1), pan-endothelial marker CD31, and blood vessel endothelium specific CD34.

Results. Vasculature showing endothelial expression of LYVE-1, D2-40, Prox-1, and CD31 in combination with absence or weak expression of CD34, as would be expected for lymphatic vessels, was seen in 11 of 25 fetuses in an age range from 14 weeks to 23 weeks of gestation (44%). This lymphatic vascular staining pattern was also observed in 4 of 15 liveborn children (27%), all within 1 month of age, of which two were born prematurely at 32 and 34 weeks of gestation. Interestingly, an incomplete lymphatic staining pattern was observed in another 4 fetuses and two liveborn children of 4 months and 7 years old. No expression of lymphatic markers was observed in adult orbital vasculature.

Conclusions. No retrobulbar intraorbital lymphatic vessels were observed in adults, however, we did observe transient expression of lymphatic markers in retrobulbar intraconal orbital vasculature during fetal and early neonatal development. The orbit may, therefore, be proposed to possess a full range of lymphatic plasticity.

INTRODUCTION

The lymphatic system is important for transportation of immune cells, interstitial fluid balance, and macromolecular absorption (lipids).[1] The organization of the lymphatic system generally parallels that of the blood vascular system, but lymphatics are not distributed as uniformly throughout the body.[1] The eyelids and conjunctiva are rich in blood vessels and lymphatics, whereas hypo vascular tissues, such as the cornea,[2] sclera,[3] and lens, have none. Other well vascularized tissues, the central nervous system, the eye with exception to the conjunctiva and limbus, and orbit, also appear devoid of lymphatics.[4-7]

Lymphangiogenesis of the orbit has been investigated extensively.[8] Previous studies of adult orbital soft tissues have reported that orbital fat lacks lymphatic vessels, but that inflammation can induce both the growth of new blood vessels and lymphangiogenesis in orbits that are inflamed[9] or in orbital infection.[10] It is thought that orbital soft tissues do not contain lymphatic vessels except for lymphatic-like structures around the dura mater surrounding the optic nerve and in the lacrimal gland.[11,12] Whether "classical" orbital lymphatics exists, therefore, is controversial.

Lymphatic vessels are derived from venous endothelial cells during embryogenesis. Whether or when the presumed orbital lymphangiogenic privilege is achieved during development is not known yet. For the developing human, it is not known whether the fetal orbit is primarily alymphatic or if there is a regression of lymphatic vessels that may transiently evolve during embryogenesis, as has been described for the murine cornea.[13]

Lymphatic vessels can be identified using a panel of immunohistochemical markers.[14-17] Endothelial markers recognize growth factors and differentiation antigens specific for lymphatic endothelial cells. It is recommended to use a minimum panel of three endothelial antibodies, which consists of one pan-endothelial marker and at least two different lymphatic endothelial-specific antibodies, to avoid misinterpretation of staining results.[14,16] Podoplanin (D2-40) is a mucin-type transmembrane glycoprotein, expressed in lymphatic endothelial cells and other cells, such as macrophages and tumor cells.[1,18] Its function includes regulation of lymphatic vascular formation and platelet aggregation. D2-40 is the most commonly used mouse monoclonal antibody against Podoplanin. Prospero-related homeobox gene-1 (Prox-1) is a nuclear transcription factor and of key importance for the development of the lymphatic system. Prox-1 is also expressed in nonendothelial cell types, such as hepatocytes, bile duct epithelium, pancreatic epithelium, cardiomyocytes, lens, retina, spinal ganglia, and vegetative ganglia.[19,20] It is not expressed in blood vascular endothelial cells, except for a small segment of the anterior cardinal vein.[21] Knockout models of Prox-1 discovered the crucial role of Prox-1 in the development of the lymphatic system.[22] Lymphatic

vessel endothelial hyaluronic acid receptor-1 (LYVE-1) is an integral membrane glycoprotein and lymphatic vessel endothelial hyaluronan receptor type 1. LYVE-1 is expressed in lymphatic, but not in blood vascular endothelium.[1] LYVE-1 may also be expressed by activated macrophages.[23] CD31 (platelet endothelial cell adhesion molecule-1, PECAM) is the most sensitive and specific pan-endothelial marker. It is an integral membrane glycoprotein, expressed on endothelial intercellular junctions, but may also be expressed by macrophages.[24] CD34 is a single-chain transmembrane glycoprotein, a hematopoietic progenitor cell antigen, and is expressed in the endothelial cells of blood vessels, but not of non-neoplastic lymph vessels.[25] CD34 may also be expressed by stromal fibroblasts.[26] Because all these markers may also be expressed in other cells than lymphatic endothelium, it is difficult to identify a lymphatic vessel based on a single marker. When multiple markers are used, lymphatic vessels will be identified with increased accuracy.

In this study, the expression of lymphatic markers is examined in the retrobulbar intraconal orbit of the developing and adult human eye with emphasis on maturation dependent changes and the possible presence of lymphatic structures within the orbit.

METHODS

Sample Selection

Human eyes were obtained by enucleation from termination of pregnancy fetuses between gestational weeks 13 and 24 (n = 25), deceased children between the ages of 0 and 15 years of age (n = 15), and adults (n = 5) as part of routine diagnostic procedures. All studies complied with the regulations of the local ethics committee. Clinical information and gestational age of fetuses and ages of the children and adults are provided in **Table 1**. Because prenatal ultrasound examination showed congenital malformations, pregnancy was terminated. The fetal eyes were harvested in case of brain malformations or with a differential diagnosis that involved syndromes that may be associated with ocular malformations. None of the diagnoses involved syndromes associated with (lymphatic) vascular malformations, like Turner-, Proteus-, Sturge-Weber-, or Klippel-Trenaunay-Weber syndrome. None of the eyes did show developmental anomalies upon macroscopic and microscopic evaluation. None of the liveborn children or adults suffered orbital infectious or inflammatory disease.

Eyes were fixated in buffered 10% formaldehyde, and pupil-optic nerve sections of 4 mm thickness were obtained after paraffin embedding. Pupil-optic nerve sections were stained for hematoxylin & eosin (H&E), and the presence of sufficient retrobulbar orbital fat for evaluation was confirmed. In addition, immunohistochemistry was performed on serial pupil-optic nerve sections.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) sections were analyzed for the presence of lymphatic vessels. Four micrometer thick sections were stained for Podoplanin (Clone D2-40, Ref.: 760-4395; Cell Marque, Rocklin, CA, USA), Prospero Homeobox-1 (Prox-1, Clone D2J6J, dilution 1:1500; Cell Signaling, Leiden, The Netherlands), Cluster Differentiation 31 (CD31, Clone JC70, Ref.: 760-4378; Cell Marque, Rocklin, CA, USA), and Cluster Differentiation 34 (CD34, Clone QBEnd/10, Ref.: 790-2927; Ventana Medical Systems, Tucson, AZ, USA) with the Ventana Benchmark Ultra automated staining system (Ventana Medical Systems). Briefly, after deparaffination the sections were processed for 32 to 64-minute antigen retrieval using Cell Conditioning Solution 1 (CC1 Ventana Ref.: 950-124). Following 32-minute incubation (16-minute for CD31) with the primary antibody at 36 deg Celsius (°C), detection was performed using the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ref.: 760-501; Ventana Medical Systems) in combination with the Amplification Kit (Ref.: 760-080; Ventana Medical Systems). Sections were counterstained with hematoxylin II (Ref.: 790-2208; Ventana Medical Systems). Due to technical limitation of the Ventana automated staining system, detection of LYVE-1 required manual interference in the protocol. For anti-LYVE-1 (Clone AF2089, dilution 1:1000; R&D Systems, Minneapolis, MN, USA) primary antibody staining was executed using the Ventana Discovery Benchmark automated staining system (Ventana Medical Systems). The following adaptations from the protocol were required: endogenous peroxidase was blocked in order to prevent unspecific signal using Inhibitor CM from the DISCOVERY ChromoMap DAB Kit (RUO) (Ref.: 760-159; Ventana Medical Systems) for 4 minutes. The secondary antibody incubation was performed with anti-Goat-HRP (Ref.:760-159; Ventana Medical Systems) for 32 minutes. Detection was executed manually with 3-amino-9-ethylcarbazoledue (AEC) diluted in 0.2M Sodium Acetate with H_2O_2. The slides were counterstained with Mayer's Hematoxylin (Cat. 4085.9005; Klinipath, Duiven, The Netherlands).

Scoring of Immunohistochemistry

Three independent reviewers scored the slides to reach consensus: a pathologist, an ophthalmologist, and a research technician with ample experience in ophthalmic pathology. Based on the presence or absence of specific signals in the orbital vasculature, lymphatic vessels were identified using a panel of immunohistochemical markers on serial sections. As described in an earlier study, a vascular structure was identified as a lymph vessel when it showed combined endothelial expression of D2-40, Prox-1, LYVE-1, and CD31 and absence or weak expression of CD34.[17] These requirements are in accordance with the first international consensus on the methodology of lymphangiogenesis quantification in solid human tumors and the consensus statement on the immunohistochemical

detection of ocular lymphatic vessels.[14,16] Lymphatic vessels of the perilimbal conjunctiva served as internal controls, external control tissue (lymph node) was applied in case of absence of conjunctival tissue.

RESULTS

Immunohistochemistry for Endothelial Lymphatic Markers

Positive endothelial staining for any of the lymphatic vascular markers was observed in the retrobulbar perioptic orbital fat of fetuses and young children. None of the adult cases showed positive endothelial staining for lymphatic markers, which is in concurrence with earlier unreported observations in enucleation specimen for uveal melanoma.[17] Positive staining for all lymphatic markers tested in combination with weak or absence of staining for CD34 was seen in 11 of 25 fetuses in an gestational age ranging from 13 to 24 weeks (44%) (**Table 1**, **Figure 1**). In another three fetuses, CD34 staining could not be evaluated with certainty because of high positive staining of perivascular orbital connective tissue. This could potentially have resulted in an underestimation of the maximum total number of positive cases (56%), see **Table 1**. Furthermore, this lymphatic vascular phenotype was observed in 4 of 15 liveborn children (27%), all within 1 month of age, of which 2 were premature born at 32 and 34 weeks of gestation, respectively (**Table 1**, **Figure 2**). The number of vascular structures that showed a lymphatic vascular phenotype varied from one to six in each case. Often a symmetrical pattern was observed showing positivity both at the nasal and temporal side of the optic nerve.

Transient Expression of Lymphatic Markers in Relation to Age

Three cases, one fetus of 13 weeks of gestation, one term born, one 4-month-old child, and one 7-year-old child, showed a vascular structure that exhibited incomplete lymphatic endothelial marker staining (**Table 1**). The 13 weeks of gestation fetus with a vascular structure staining positive for LYVE-1, Prox1, and CD31 was a termination of pregnancy because of the combination of omphalocele and encephalocele. CD34 could not be evaluated in this case. The 4-month-old child with a vascular structure positive for LYVE-1, D2-40, CD31, and CD34 died of hemophagocytic lymphohistiocytosis, which was not present in the orbital tissues. The 7-year-old child had a vascular structure that stained positive for LYVE-1 in combination with CD31 and CD34 positive staining. This implies a transient expression of lymphatic markers and that Prox-1 may the first marker to be lost followed by D2-40 and LYVE 1. Moreover, for second trimester fetuses, a lymphatic vascular pattern was observed in a maximum of 14 of 25 cases (56%, including

those cases where CD34 could not be evaluated), whereas in third trimester premature and term born children, such a complete pattern was only observed in 4 of 15 (27%) cases. No lymphatic vascular pattern was found in adults, also indicating a progressive loss of expression of lymphatic markers with age.

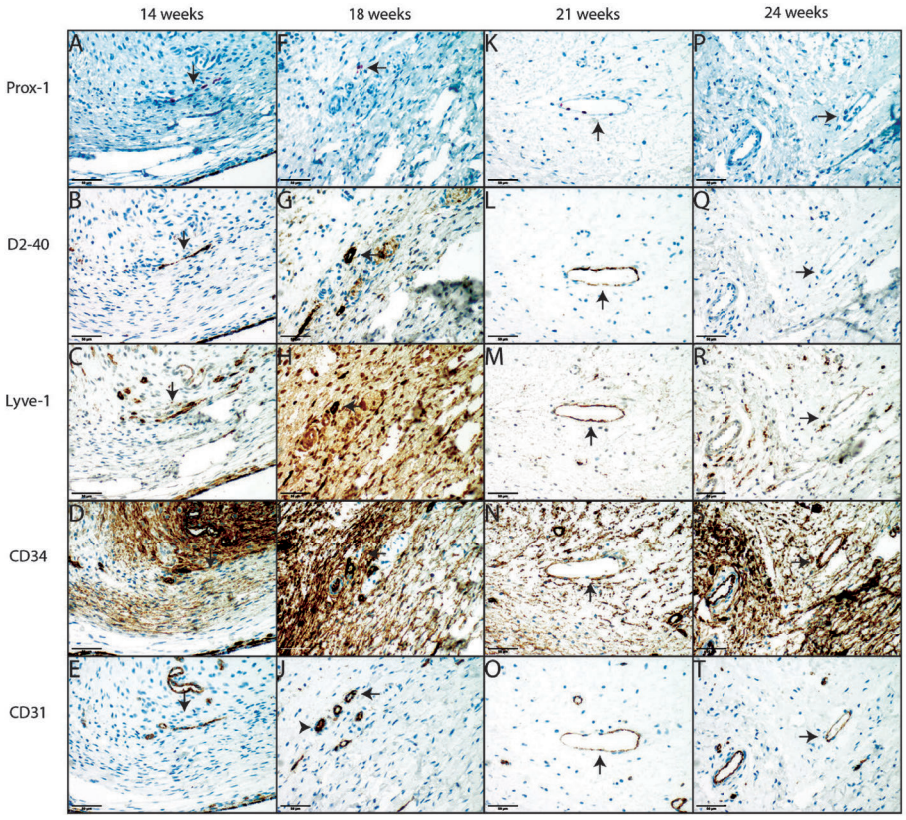

Figure 1. Overview of identification of lymphatic vessels with Prox-1, D2-40, LYVE-1, CD34, and CD31 in second trimester fetuses. Lymphatic phenotype staining pattern of a 14-week-old fetus (**Table 1**, case 2) is shown by a positive endothelial staining for Prox-1, D2-40, and LYVE-1 (**A**, **B**, **C**, respectively) combined with a weaker staining for CD34 compared to the surrounding blood vessel endothelium. (**D**) Positive staining for CD31. (**E**) Lymphatic staining pattern of an 18-week-old fetus (**Table 1**, case 8) showed positive endothelial phenotype staining for Prox-1, D2-40, and LYVE-1 (**F**, **G**, **H**) combined with a negative staining for CD34 (**I**) and a positive staining for CD31. (**J**) Lymphatic phenotype staining pattern of a 21-week-old fetus (**Table 1**, case 11) showed positive endothelial staining for Prox-1, D2-40, and LYVE-1 (**K**, **L**, **M**, respectively) combined with a weak staining for CD34. (**N**) and a positive staining for CD31. Note the asterisk (*) showing strong positive staining in blood vessel endothelium for CD34 as reference. (**O**) Staining pattern of vasculature in a 24-week-old fetus (**Table 1**, case 25) showed negative staining for Prox-1, D2-40, and LYVE-1 (**P**, **Q**, **R**, respectively) combined with a positive staining for CD34 (**S**) and a positive staining for CD31. (**T**) No lymphatic phenotype staining pattern was observed in this case.

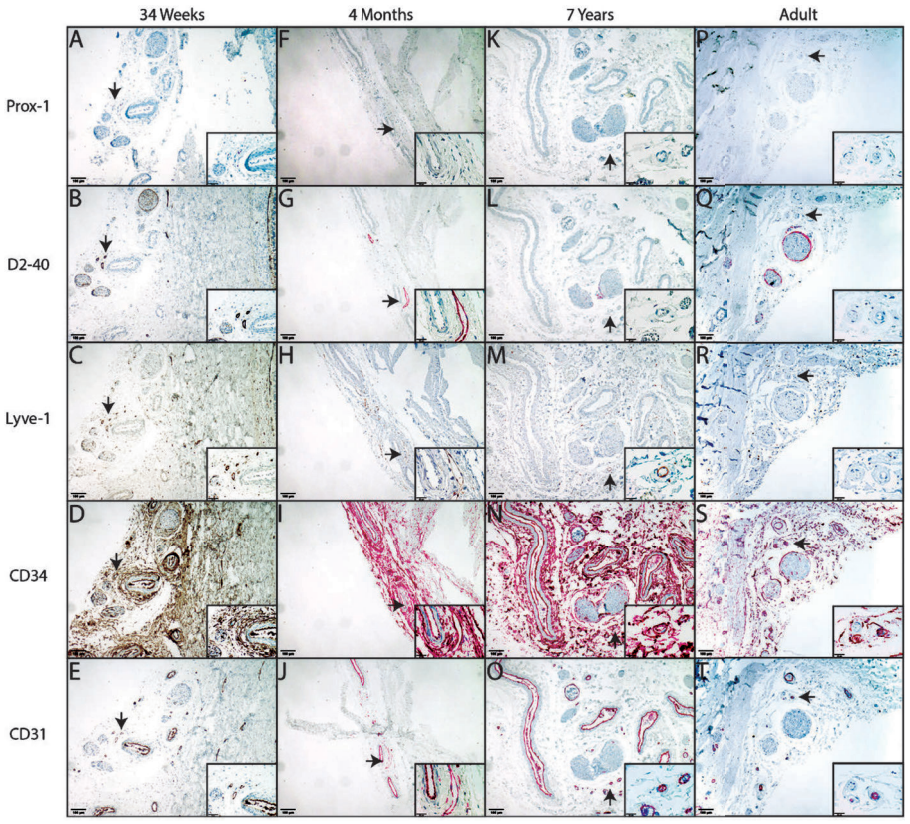

Figure 2. Overview of identification of lymphatic vessels with Prox-1, D2-40, LYVE-1, CD34, and CD31 in third trimester and older children and adult. Lymphatic phenotype staining pattern of a 14-week-old premature born child at 34 weeks of gestation (**Table 1**, case 29) is shown by a positive staining for Prox-1, D2-40, and LYVE-1 (**A**, **B**, **C**, respectively) combined with a negative staining for CD34 (**D**) and a positive staining for CD31. (**E**) Incomplete lymphatic staining pattern of a 4-month-old child showed positive staining for LYVE-1 and D2-40 (**G**, **H**), but a negative staining for Prox-1. (**F**) Whereas CD34 and CD31 both show positive staining. (**I**, **J**) Incomplete lymphatic staining pattern of a 7-year-old child showed positive staining for LYVE-1 (**M**), but negative staining for Prox-1 and D2-40. (**K**, **L**) CD34 and CD31 both showed positive staining. (**N**, **O**) Vascular staining pattern of an adult showed no positive staining of Prox-1, D2-40, and LYVE-1 (**P**, **Q**, **R**), but did show positive staining for CD34 and CD31. (**S**, **T**) No lymphatic phenotype staining pattern was observed in adult cases. Large panels: original magnification 50x. Insets: original magnification 200x.

Transient Expression of Lymphatic Markers in Relation to Age

Three cases, one fetus of 13 weeks of gestation, one term born, one 4-month-old child, and one 7-year-old child, showed a vascular structure that exhibited incomplete lymphatic endothelial marker staining (**Table 1**). The 13 weeks of gestation fetus with a vascular structure staining positive for LYVE-1, Prox1, and CD31 was a termination of pregnancy because of the combination of omphalocele and encephalocele. CD34 could not be evaluated in this case. The 4-month-old child with a vascular structure positive for LYVE-1, D2-40, CD31, and CD34 died of hemophagocytic lymphohistiocytosis, which was not present in the orbital tissues. The 7-year-old child had a vascular structure that stained positive for LYVE-1 in combination with CD31 and CD34 positive staining. This implies a transient expression of lymphatic markers and that Prox-1 may the first marker to be lost followed by D2-40 and LYVE 1. Moreover, for second trimester fetuses, a lymphatic vascular pattern was observed in a maximum of 14 of 25 cases (56%, including those cases where CD34 could not be evaluated), whereas in third trimester premature and term born children, such a complete pattern was only observed in 4 of 15 (27%) cases. No lymphatic vascular pattern was found in adults, also indicating a progressive loss of expression of lymphatic markers with age.

Table 1. Description of the cases with causes of death

Case nr	Age	Cause of death / abortion	LYVE-1	Prox-1	D2-40	CD31	CD34
1	13+3 weeks	Omphalocele and encephalocele	+	+	-	+	N/A
2*	14+0 weeks	Steinfeld syndrome	+	+	+	+	w
3	16+1 weeks	Osteogenesis imperfecta type II	+	+	+	+	-
4	16+4 weeks	Holoprosencephaly with MCA	-	-	-	+	+
5	16+5 weeks	Arthrogryposis	+	+	+	+	-
6	17+0 weeks	Joubert syndrome	-	-	-	-	-
7	18+5 weeks	Isolated lumbosacral myelomeningocele	+	+	+	+	-
8*	18+6 weeks	Premature rupture of membranes	+	+	+	+	-
9	21 weeks	Ellis van Creveld syndrome	-	-	-	+	+
10	21 weeks	MCA no syndrome diagnosis	+	+	+	+	N/A
11*	21+2 weeks	MCA no syndrome diagnosis	+	+	+	+	w
12	21+3 weeks	Aqueductal stenosis	+	+	+	N/A	N/A
13	21+4 weeks	Aqueductal stenosis	+	+	+	+	_
14	21+4 weeks	Osteogenesis imperfecta	-	-	-	+	+
15	22+4 weeks	Vermis and callosal hypoplasia	-	-	-	+	+
16	22+4 weeks	Walker Warburg syndrome	-	-	-	+	+
17	22+5 weeks	Isolated corpus callosum agenesis	+	+	+	+	-
18	22+6 weeks	IUGR due to maternal pre-eclampsia	-	-	-	+	+
19	23+0 weeks	MCA no syndrome diagnosis	+	+	+	+	-
20	23+1 weeks	Isolated ventriculomegaly	-	-	-	+	+
21	23+3 weeks	TUBB2B gene mutation	+	+	+	+	-
22	23+3 weeks	Unexplained hydrops foetalis	+	+	+	N/A	N/A
23	23+5 weeks	Isolated complex cardiac malformation	+	+	+	+	-
24	24 weeks	Isolated midline arachnoidal cyst	-	-	-	+	+
25*	24+3 weeks	Diaphragmatic hernia	-	-	-	+	+
26	27+2 weeks 6 d old	Perinatal death, Goldenhar syndrome	-	-	-	+	+
27	32+6 weeks 2 d old	Perinatal death, diaphragmatic hernia	+	+	+	+	-
28	33+5 weeks 5 d old	Perinatal death, lissencephaly spectrum	-	-	-	+	+
29#	34+5 weeks	Hydrocephalus	+	+	+	+	-
30	1 day old	Perinatal death, abusive head trauma	+	+	+	+	-
31	8 days old	Chondrodysplasia punctate	+	+	+	+	-
32	6 weeks	Abusive head trauma	-	-	-	+	+
33	7 weeks	Abusive head trauma	-	-	-	+	+
34	4 months	Abusive head trauma	-	-	-	+	+
35	4 months#	Hemophagocytic lymphohistiocytosis	+	-	+	+	+
36	6 months	Pneumonia	-	-	-	+	+
37	7 months	Abusive head trauma	-	-	-	+	+
38	12 months	Endomyocarditis	-	-	-	+	+
39	7 years#	Bronchopneumonia	+	-	-	+	+
40	15 years	Ketoacidosis	-	-	-	+	+
41	32 years	Decompensatio cordis	-	-	-	+	+
42#	53 years	Choroidal melanoma	-	-	-	+	+
43	54 years	Unknown (eye bank specimen)	-	-	-	+	+
44	74 years	Bronchopneumonia	-	-	-	+	+
45	68 years	Bronchopneumonia	-	-	-	+	+

The last five columns represent the staining pattern per individual case. Cases that could not be investigated using the full panel of markers due to lack of tissue, technical issues during staining or couldn't be interpreted with confidence are indicated as not assessable (N/A). The cases illustrated in **Figure 1** have been highlighted with an *, the cases illustrated in **Figure 2** have been highlighted with an #. One adult case was obtained due to enucleation of uveal melanoma. IUGR = intrauterine growth retardation; MCA = multiple congenital malformations; w = weak expression

DISCUSSION

This study examined the presence of retrobulbar intraorbital lympatic vessels during fetal and neonatal development and in adults by using a panel of lymphatic markers on enucleation specimen. Our panel of markers was designed to distinguish lymphatic vessels from blood vessels. Our criteria to identify lymphatic vessels also relies on the absence or weak expression of CD34, which should be carefully interpreted in early development and adulthood. During early development, CD34 is expressed in mesenchymal cells,[26] hematopoietic progenitor cells,[27] and developing vasculature.[28] Furthermore, endothelial cell-fate during early development is much less understood, which makes identification of lymphatic vessels challenging in embryonic tissue. Whereas in adulthood, CD34 can be occasionally and irregularly expressed in lymphatic vessels depending on the type of vessel, localization, and tissue, albeit in a much lower staining intensity when compared to blood vessels.[29,30] When identifying lymphatics in neural-rich tissue, such as the eye, one needs to be aware of positive staining of D2-40[31] and CD34[32] in nerve sheath cells. Especially in an immunofluorescent approach, this can mimic a vascular structure, and, thus, may appear as a lymphatic vessel. With the use of a broader lymphatic marker panel, these structures can be identified as nonlymphatic structures by lack of LYVE-1, Prox-1, and CD31 (data not shown). We could not detect retrobulbar intraorbital lymphatic vasculature in adult samples using five specific markers. This is in concordance with earlier unreported observations, when 16 adult eyes were examined for lymphatic vessel recruitment in uveal melanoma.[17] However, we did find transient expression of lymphatic markers in retrobulbar orbital vasculature during fetal and early neonatal development. Others did not observe intraorbital lymph vessels in four 10 to 12 weeks of gestation old fetuses that were serially sectioned.[33] Although, in that study, only Podoplanin was used as an immunohistochemical marker and our cases are of more advanced gestational age. The use of multiple markers in the current study may explain the increased likelihood for identification of lymphatic vasculature. The fact that not all cases from a similar gestational age proved to be positive may be explained by a potential for under detection because a limited amount of orbital fat was available in the enucleation specimen. Complete removal, embedding, and sectioning of the orbital contents may be expected to increase the likelihood for identification of such vessels in future. Because such a procedure would not be justified as part of a diagnostic procedure, a separate specific parental consent would be required. The observation that, in the current study, expression of lymphatic markers decreases with (gestational) age, made us hypothesize that this is most likely a transient developmental phenomenon that does not signify consistent orbital lymphangiogenesis. These findings are similar to what has been reported for the murine cornea.[13] That study demonstrated that the mouse cornea was endowed with a significant number of lymphatic vessels

that underwent spontaneous formation and regression during a critical period after birth, which was not observed for blood vessels. Lymphatic growth can be reactivated in the adult cornea and orbit after inflammatory stimulation. The transient expression of lymphatic vessel markers in retrobulbar orbital vessels during fetal and early neonatal development may, therefore, share overlapping features with lymphatic vessel growth and regression during postnatal development in the mouse model. In line with what was described for the murine cornea, it could be speculated that the lymphatic status of the orbit is orchestrated and maintained by a similar combination of pro- and antilymphatic factors already known or yet to be discovered. Certain physiologic or pathologic stimulations will tip the balance in favor of lymphatic formation or regression.[13] This may also explain previous studies of adult orbital soft tissues that have reported that orbital fat lacks lymphatic vessels, but that inflammation can induce both the growth of new blood vessels and lymphangiogenesis in orbits that are inflamed or in orbital infection.[9,10]

In conclusion, this study describes that transient developmental expression of lymphatic markers is a feature observed in retrobulbar intraconal vasculature during the fetal and early neonatal period. The orbit may, therefore, be proposed to possess a full range of lymphatic plasticity.

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CHAPTER 5

HIGHER PERCENTAGE OF FISH-DETERMINED MONOSOMY 3 AND 8q AMPLIFICATION IN UVEAL MELANOMA CELLS RELATE TO POOR PATIENT PROGNOSIS

Thomas van den Bosch
Jackelien van Beek
Jolanda Vaarwater
Robert Verdijk
Nicole Naus
Dion Paridaens
Annelies de Klein
Emine Kiliç

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ABSTRACT

Purpose. To investigate the relation between patient survival and incrementally increasing percentages of fluorescence in situ hybridization–determined complete loss of chromosome 3 (monosomy 3) and gain of chromosome 8q in primary uveal melanoma cells.

Methods. Clinicopathological factors were related to disease-free survival. Fluorescence in situ hybridization was performed using probes on chromosomes 1, 3, 6, and 8. The percentages of UM cells with monosomy 3 or chromosome 8q gain were classified in groups with incrementally increasing percentages and related to disease-free survival. Correlations between clinical factors and cytogenetic aberrations were also analyzed.

Results. Two-hundred twenty choroidal and ciliary body melanomas were analyzed. The following proved to be significant predictors of survival in univariate analysis: older patient age ($P = 0.003$); large tumor diameter ($P < 0.001$); mixed cell type ($P = 0.001$); presence of closed microvascular loops ($P < 0.001$); loss of chromosome 1p ($P = 0.006$); monosomy 3 ($P < 0.001$); gain of 6p ($P < 0.001$); and gain of chromosome 8q ($P < 0.001$). Multivariate Cox analysis displayed monosomy 3 (Hazard ratio [HR] 2.83, $P = 0.002$) and gain of chromosome 8q (HR 3.13, $P = 0.002$) as the most important independent prognostic factors of poor survival, followed by older patient age (HR 1.02, $P = 0.017$). Increasing percentages of monosomy 3 and gain of chromosome 8q in tumor cells showed a correlation with worse prognosis (Log-rank test 49.9 and 40.4, both $P < 0.001$) and increased number of additional copies of 8q correlated with shorter disease-free interval (Log-rank test 45.7, $P < 0.001$).

Conclusions. A high percentage monosomy 3 and chromosome 8q gain in primary UM cells showed a strong relation with poor disease-free survival compared with low percentage aberrations.

INTRODUCTION

Uveal melanoma is the most common primary intraocular malignancy in adults with an annual incidence of 5–7 cases per million. Nearly half of all patients with UM eventually die of metastases which are most often late-appearing.[1,2] In search for prognostic factors, several clinical, pathological, and genetic parameters have been identified. Genetic factors have proven to be the most significant factors of all and can reliably indicate high risk of metastasis and poor survival in patients with UM.[3,4] Nonrandom chromosomal alterations are present in more than 80% of cases with most frequently a complete loss of chromosome 3 (monosomy 3) in 50% of cases. Monosomy 3 is the most important chromosomal factor relating to a 4-year overall survival of only 30%.[5,6] Other known but less frequently occurring alterations that also relate with prognosis are gain of chromosome 6p or 8q, loss of chromosome 1p or 6q, and co-occurrence of chromosome 1p and 3 loss.[5,7-9]

Fluorescence in situ hybridization is a reliable technique for assessing chromosomal aberrations in UM; therefore, many large referral centers routinely use fluorescence in situ hybridization (FISH) for the analysis of the chromosome 3 status of a tumor. FISH enables in situ analysis of chromosomal aberrations in tumor cells and by using a cutoff threshold for identification of loss, tumors are either classified as chromosome 3 disomic or monosomic. By classifying tumors in one of these groups using a cutoff threshold, information on exact percentages of aneuploidy and their possible relation to prognosis is disregarded. Using FISH analysis, this study assessed the percentages of tumor cells with loss of chromosome 3 or gain of chromosome 8q for each tumor separately and correlated these findings with patient prognosis. Tumors were classified according to the FISH counts in groups of incrementally increasing percentages of chromosome 3 or 8q aneuploidy: 15%–33% (10%–33% for gain of chromosome 8q), 33%–66%, and 66%-100%, and investigated whether a high percentage of aneuploidy in the tumor is related to a decreased survival. If so, this could provide a more precise prognosis for patients with low, intermediate, and high percentages of chromosome 3 or 8q aneuploidy in their tumor and could be used for selecting patients eligible for adjuvant therapy.

METHODS

Between July 1994 and November 2010, tumor material was collected from 248 patients who underwent enucleation for UM. Thirteen iris melanomas and fifteen hyperaneuploid cases were excluded from this study because of the differences in molecular behavior.[10,11] Routine clinical systemic evaluation including blood liver function tests was conducted before enucleation was performed. Fresh tumor tissue was harvested within 1 hour after enucleation of the remaining 220 ciliary-body and choroidal melanomas, and was processed for histopathologic

and genetic research. Histopathologic examination was conducted according to standardized protocols and confirmed the origin of the tumor as well as tumor size, cell type, and presence of microvascular patterns (closed vascular loops). Informed consent was obtained prior to enucleation and the study was performed according to guidelines of the Declaration of Helsinki. Clinical data and follow-up data regarding metastases and tumor-related death were obtained from medical records and by contacting the general physician. In total, three patients were lost to follow-up: the first patient was 57 years old when he was lost to follow-up after 28 months because he moved abroad to an unknown destination. The second patient was 89 years old and was lost to follow-up after 69 months; the third patient was 93 years old and lost to follow-up after 18 months. These three patients had no sign of metastasis at the last follow-up moment.

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization allows interphase cytogenetic analysis of fresh or archival tumor tissue by using differentially labeled fluorescent probes mapping to specific chromosomal regions. With this technique, copy number alterations can be determined in a large number of cells. Fresh tumor tissue from enucleated eyes containing UM was routinely used for direct interphase FISH (chromosome 1, 3, 6, and 8) as described previously.[12] The following probes were used: RP11-48E9 (1p36); RP11-384L8 (3p22) or RP11-522N9 (3p13); Pα3.5 (centromere 3); YAC 827D3 (3q24); RP11-356B3 (6p22); RP11-787I22 (6q21); RP11-24P4 (8p21); D8Z2 (centromere 8); and RP11-88J22 (8q22). After optimization, probe YAC827D3 (3q24) was replaced with probe RP11-64F6 (3q25), which was used for tumor samples collected from December 2000. If FISH on fresh tissue failed, due to insufficient availability of fresh tumor tissue or technical reasons, FISH was carried out on paraffin sections of the tumor (archival tissue) as described before.[11] In all cases, up to 300 cells were counted according to the criteria of Hopman.[13] Cutoff limits for FISH on fresh and archival tissue were adopted from available literature.[14,15] The cutoff limit used for amplification was 10% for FISH on fresh tissue and paraffin tumor sections. Cutoff limit for deletion with FISH on fresh tumor tissue was 15%; and for paraffin tumor sections, the cutoff limit was corrected for by a reference probe on chromosome 5[11] and defined as 25%. As cutoff limits for deletion differed by 10% for paraffin sections and fresh tumor tissue, paraffin section counts were adjusted by subtracting 10%, thereby allowing easy comparison.

After adjustment of the cutoff limit for deletion, the following groups were defined for tumors with monosomy 3: low percentage (15%–33%), intermediate percentage (33%–66%), and high percentage monosomy 3 (66%–100%). Due to the 10% cutoff limit for amplification, tumors with chromosome 8q amplification (gain) were

classified as low percentage (10%–33%); intermediate percentage (33%–66%); or high percentage gain of chromosome 8q (66%–100%).

Chromosome 3 and chromosome 8q counts were classified in groups of 33% as this resulted in the most reliable comparisons with the intermediate groups, which had a low number of cases. Chromosome 3 status was assessed by a 3p probe, centromere 3 probe, and a 3q25 probe, to allow for assessment of monosomy of chromosome 3. If the chromosome 3 probes showed a difference in percentage of tumor cells with deletion, the highest percentage was used for further analysis. Chromosome 8q alterations were evaluated separately by one probe on 8q21 and no specific cutoff correction was made for using paraffin sections as truncation and cutting-artifacts are not a major issue for cells showing more than two signals.

Statistical Analysis

The influence of single prognostic factors on metastasis-free survival was assessed by using univariate Log-rank analysis or Cox proportional hazards analysis. Cox regression multivariate analysis was performed to identify the independent value of the prognostic factors. Kaplan-Meier survival analyses were performed for estimation of survival probabilities with metastasis or death due to metastasis as primary clinical endpoint. Kaplan-Meier curves were computed for patients overall, and with incrementally increasing percentages of monosomy 3, and chromosome 8q gains. Mann-Whitney tests were used to assess correlations between different prognostic factors. All P values were two-sided and significance was set at $\alpha = 0.05$. The analyses were performed using the statistical software SPSS Version 17.0 (SPSS Inc., Chicago, IL).

RESULTS

In total, 220 UMs were successfully analyzed with interphase FISH on fresh tumor tissue in 189 cases, and in 31 cases with additional FISH on paraffin tumor sections. Using both tissue types, chromosome 3 status was successfully assessed in all 220 cases and chromosome 8q status in 201 cases (91%). Monosomy 3 was present in 12 of 19 tumors where data on chromosome 8q status was missing. Co-occurrence of monosomy 3 and gain of chromosome 8q was present in 102 of 201 cases (51%); monosomy 3 without gain of 8q was present in 20 cases (10%); and gain of 8q without monosomy 3 was present in 32 cases (16%). Of the 220 patients recruited for this study,121 were male. The mean age of all patients was 62 years (median 62 years, range 21–87 years). The mean duration of follow-up, from diagnosis to end of study, was 4.7 years (range 0.3–15.9 years), with metastases occurring at

a mean follow-up of 3.1 years (range 0.3–11.0 years). Eighty-one patients died from metastatic disease and five were diagnosed with metastases at the time of evaluation.

Univariate analysis of the single prognostic risk factors showed a significantly decreased survival for patients with UM with the presence of epithelioid cells, closed vascular loops, loss of chromosome 1p, monosomy 3, and gain of chromosome 8q (**Table 1**). Large tumor diameter and older patient age were significantly related to poor survival as well. A gain of chromosome 6p was related to a more favorable prognosis.

Table 1. Univariate analysis of prognostic markers on disease-free survival in 220 uveal melanomas

Variable	Mean, median (range)	No. of patients (%)	Missing data (%)	P Value*†
Age at diagnosis	62 yrs, 62 (21–87)		–	0.003
Largest tumor diameter	12.8 mm, 13.0 (2–30)		–	<0.001
Tumor height	7.5 mm, 7.0 (1–22)		–	0.178
Male gender		121 (55)	–	0.218
Mixed/epithelioid cell type		154 (70)	–	0.001
Involvement ciliary body		30 (14)	4 (2)	0.065
Closed vascular loops		90 (41)	15 (7)	<0.001
Loss of chromosome 1p		66 (30)	–	0.006
Monosomy 3		134 (61)	–	<0.001
Gain of chromosome 6p		93 (42)	26 (12)	<0.001
Loss of chromosome 6q		65 (30)	33 (15)	0.332
Gain of chromosome 8q		134 (61)	19 (9)	<0.001

* Cox-regression analysis

† Log-rank test

Kaplan-Meier survival analysis displayed poor survival probabilities for patients having tumors with monosomy 3 (Log-rank test 36.5, $P < 0.001$) (**Figure 1a**). Survival probabilities were even worse if the patients' tumors had a high percentage of monosomy 3 in analyzed cells, and Kaplan-Meier survival analysis showed a significantly worse survival for patients with high percentage of monosomy 3 in their tumor compared with patients having tumors with low or intermediate percentage monosomy 3 (Log-rank test 49.9, $P < 0.001$) (**Figure 1b**). The disomy 3 group was analyzed next to medium and high percentage aneuploidy groups as well and displayed a more favorable prognosis than the higher percentage aneuploidy groups (**Figure 1c**). Presence of chromosome 8q gain in tumors also

correlated with worsening patient survival (Log-rank test 31.6, P < 0.001) (**Figure 1d**). The high and intermediate percentage gain of chromosome 8q groups displayed comparable survival probabilities, which were worse than with low percentage gain (Log-rank test 40.4, P < 0.001) (**Figure 1e)**. The disomy 8q group was analyzed next to higher percentage aneuploidy groups as well, displaying a more favorable prognosis (**Figure 1f**).

Cox proportional hazard analysis was performed with all factors that were significant after univariate analysis to exclude confounding variables and identify the independent prognostic value of chromosome 3 and 8q in this cohort. Older age, monosomy 3, and gain of 8q proved to be independent negative prognostic factors. If these factors were stratified for the different age groups and increasing percentages of monosomy 3 or gain of chromosome 8q, the highest age group and highest percentage tumor aneuploidy groups showed the strongest correlation with poor survival (**Table 2**). General prognostic factors such as tumor diameter, epithelioid cell type, presence of closed vascular loops, loss of chromosome 1p, and gain of 6p lost significance after multivariate analysis.

Table 2. Multivariate analysis of prognostic markers on disease-free survival in 220 uveal melanoma patients

Variable	Hazard Ratio	95% CI	P Value
Age at diagnosis*†	**1.021**	**1.004–1.039**	**0.017**
<40 y	1.507	0.607–3.743	0.377
40–50 y	1.636	0.928–2.882	0.089
50–60 y	1.450	0.918–2.290	0.111
60–70 y	1.201	0.759–1.901	0.434
>70 y	3.064	1.387–6.771	0.006
Loss of chromosome 3‡	**2.832**	**1.488–5.388**	**0.002**
Low percentage (15%–33% cells), *n* = 9	1.907	0.538–6.769	0.318
Intermediate percentage (33%–66% cells), *n* = 16	2.004	0.688–5.841	0.203
High percentage (66%–100% cells), *n* = 109	5.877	3.191–10.825	<0.001
Gain of chromosome 8q‡	**3.131**	**1.538–6.375**	**0.002**
Low percentage (10%–33% cells), *n* = 12	0.923	0.193–4.418	0.920
Intermediate percentage (33%–66% cells), *n* = 18	5.720	2.190–14.941	<0.001
High percentage (66%–100% cells), *n* = 104	6.139	3.035–12.420	<0.001

95% CI = 95% Confidence Interval

* Cox proportional hazard analysis

† Per year increase

‡ Likelihood ratio test

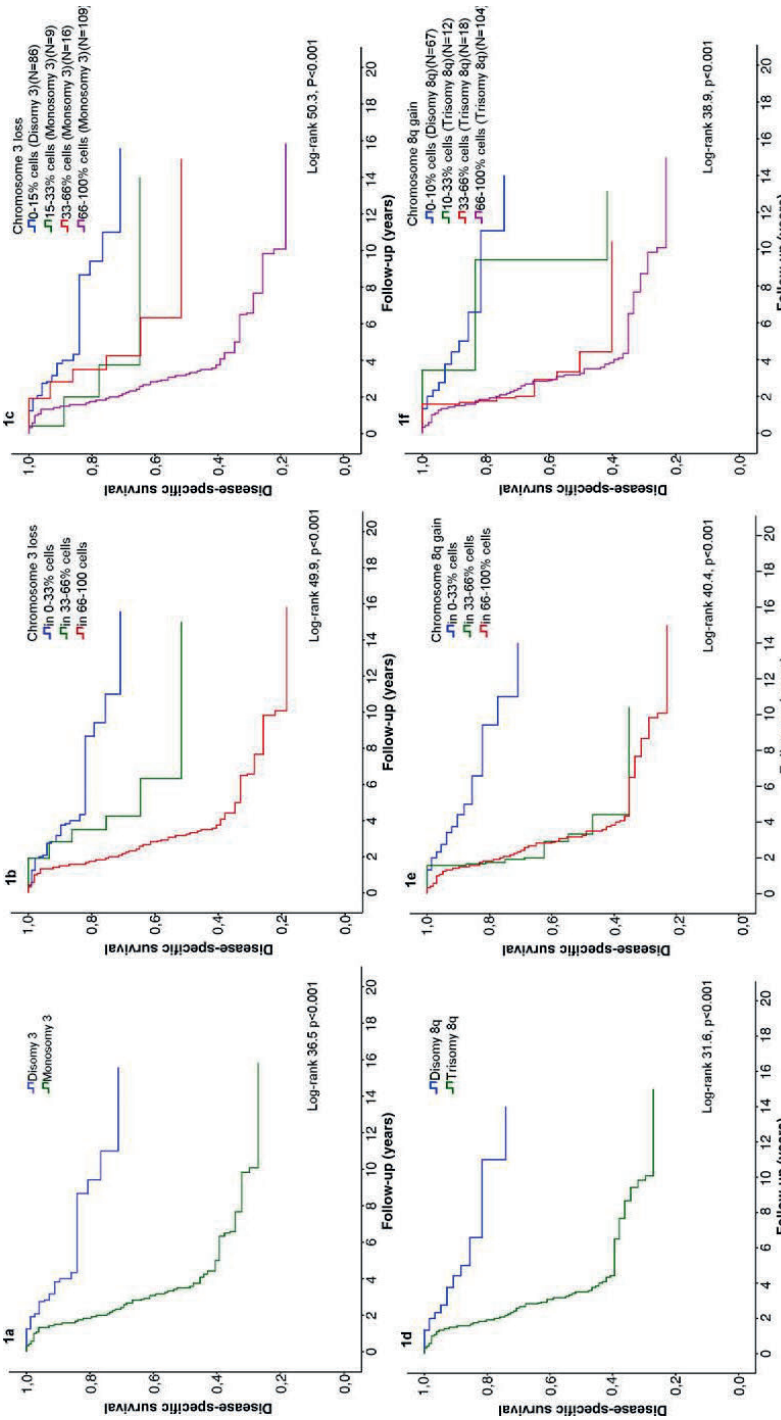

Figure 1. Kaplan-Meier survival curves. (**a**) Chromosome 3 status. (**b**) Chromosome 3 loss (illustrates incrementally increasing percentages). (**c**) Chromosome 3 status (illustrates increasing percentages). (**d**) Chromosome 8q status. (**e**) Chromosome 8q gain (illustrates incrementally increasing percentages). (**f**) Chromosome 3 status (illustrates increasing percentages).

Tumors with high percentage monosomy 3 were larger in diameter than tumors with low percentage monosomy 3 (P = 0.028) (**Table 3**). The correlation between high percentage gain of chromosome 8q and large tumor diameter was slightly stronger than for monosomy 3 (P = 0.024). There was no direct correlation found between older patient age and larger tumor size. Tumors with monosomy 3 and a high percentage of chromosome 8q gain frequently had additional copies of chromosome 8q present (**Figure 2**), which related to worse patient survival (**Figure 3**, Log-rank test 45.7, P < 0.001). Moreover, an increased number of additional copies of chromosome 8q related to a shorter disease-free interval.

Table 3. Correlation between tumor size and abnormalities of chromosome 3 and 8q

Clinical Data	Chromosome 3 loss			Chromosome 8q gain		
	15%–33% Cells	66%–100% Cells	P Value	10%–33% Cells	66%–100% Cells	P Value
Mean tumor diameter (mm)	12.2	13.4	0.028	11.8	13.8	0.024
Mean tumor thickness (mm)	7.5	8.0	0.341	6.5	7.9	0.154

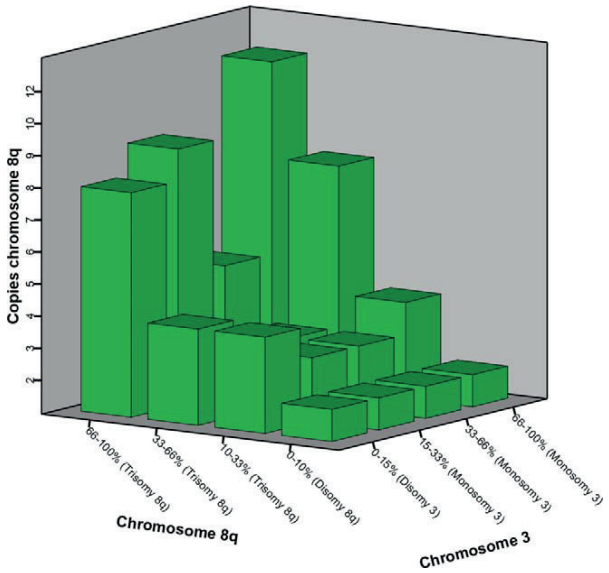

Figure 2. 3-D chart showing FISH counts of chromosome 3 and 8q according to chromosome 8q copy number.
X-axis: classification according to the percentage of tumor cells with gain of chromosome 8q; 0%–10% (disomy 8q); 10%–33% (low percentage gain); 33%–66% (intermediate percentage gain); 66%–100% (high percentage gain).
Y-axis: number of copies of chromosome 8q.
Z-axis: classification according to the percentage of tumor cells with monosomy 3; 0%–15% (disomy 3); 15%–33% (low percentage monosomy); 33%–66% (intermediate percentage monosomy); 66%–100% (high percentage monosomy).

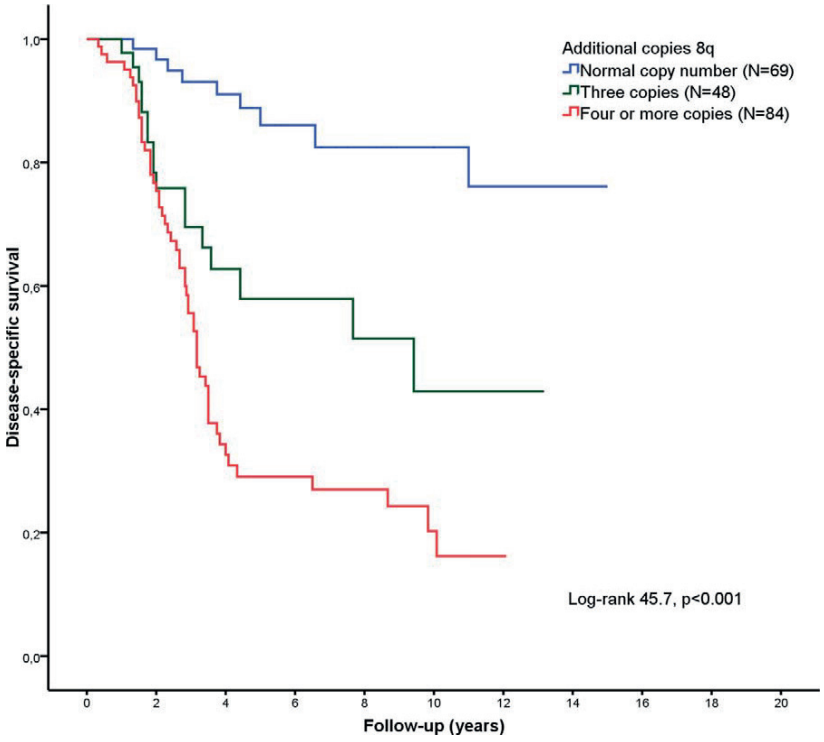

Figure 3. Kaplan-Meier survival curves according to copies of chromosome 8q present.

DISCUSSION

Study findings confirm the importance of monosomy 3 as an important prognostic cytogenetic factor for metastatic disease in UM. Additionally, gain of chromosome 8q and older patient age were classified as important independent prognostic factors in this study. Our FISH results show a gradually worsening patient prognosis for UMs with incrementally increasing percentages of monosomy 3. A higher percentage gain of chromosome 8q (more than 33% of tumor cells) correlates more with worsening survival than low percentage 8q gains (in 10%–33% of tumor cells), while the intermediate and high percentage 8q aneuploidy groups display comparable patients' survival (**Figure 1**). High percentage monosomy 3 in our study relates to a 4-year overall survival probability of 15%–20% (**Figures 1b**, **1c**). This is comparable to or slightly worse than the reported 4-year overall survival of 30% for monosomy 3 tumors in general.[5,6] Also in the low percentage monosomy 3 group, patients had an increased risk of metastasis compared with the disomy 3 group (HR = 1.9) (**Table 2**); and 3 out of 9 patients from the low percentage

group died from metastasis after a mean follow-up of 2.1 years (range 0.4–3.8 years). With a longer follow-up, even more patients may develop metastases. Interestingly, these patients with metastases all had low percentage monosomy 3 next to a high percentage gain of chromosome 8q. **Figure 2** illustrates that a low percentage of monosomy 3 may coincide not only with higher percentages of gain of chromosome 8q, but also additional copies of chromosome 8q in tumor cells. This supports previous results of Sisley *et al.*[16] where additional copies of chromosome 8q predicted a worse disease-free survival.

Considering polysomy 8q, low percentage gain showed borderline favorable prognosis after multivariate analysis (HR = 0.923), which is remarkable as all other aneuploidy groups are correlated with poor prognosis. This result could be due to the small number of patients in this group and may, therefore, need more cases to allow for a reliable estimation of the risk of metastasis for this group. At present, 2 out of 12 patients from this group of low percentage gain of chromosome 8q died from metastasis. Of the remaining patients within this group, eight have a tumor with simultaneous loss of chromosome 3 and four patients have a follow-up of less than 2 years. Considering this, these patients might develop metastasis in the near future too.

Co-occurrence of chromosome 3 and 8 was reported before[16,17] and may be referred to as genetic imbalance, as stated by Patel *et al.*[18] Current study findings indicate the importance of determining chromosome 8q status when there is no monosomy 3 or only low percentage monosomy 3 in a tumor, as this may coincide with a high percentage gain of chromosome 8q (or increased copies of 8q) and lead to worsening patient survival. This hypothesis is also supported by Patel *et al*.[18] where a genetic imbalance (monosomy of chromosome 3, gain of chromosome 8, or both) was associated with worsening survival. However, the cutoff limits in the study by Patel *et al.*[18] were 30% due to the sensitivity of the probes used. The authors report two patients with genetic imbalance in 20% of tumor cells who survived for over 100 months, but also hypothesized that these patients might develop metastases in the long run, and that minimal genetic imbalances (of 5%–10%) could lead to development of metastatic disease. Bronkhorst *et al.*[19] also report that tumors with 5% of monosomy 3 correlate to a high risk of metastatic disease using a centromere probe. However, in their study, they state that a threshold of 30% for monosomy 3 predicts high risk of metastasis more accurately. Even though the low and intermediate percentage aneuploidy groups in our study were small, still a significant number of patients with monosomy in less than 30% of tumor cells died due to metastasis. Therefore, using a threshold of 30% and higher for monosomy 3 will not lead to identification of these high-risk patients who would consequently be excluded from any adjuvant treatment.

In contrast to monosomy 3, chromosome 8 alterations are known to be a late event in UM development, relating to large tumor size.[20] This relation with tumor size was confirmed with the present study as high percentage chromosome 8q gain in tumor cells related to a larger tumor diameter than low percentage 8q gain (**Table 3**). The high percentage gain of chromosome 8q group also frequently showed increased copy numbers of 8q (**Figure 2**), which in turn correlated with reduced patient survival and shorter disease-free interval (**Figure 3**). This could indicate that when UMs grow larger, cytogenetic alterations accumulate in an increasing number of cells, leading to additional copies of chromosome 8q and worsening survival. On the other hand, presence of cytogenetic alterations in tumor cells may give the tumor a growth advantage.

It remains a major issue whether actual percentages of aberrations found in analyzed tumor sections reflect the situation in all parts of the tumor. Several groups already reported intra-tumor heterogeneity to be present in UM,[11,21,22] and biopsy taking may therefore result in sampling error. However, discordance of chromosome 3 results was only found in a minority of cases analyzed by fine needle aspiration biopsy specimens and direct single-cell suspensions[12] and paraffin sections of different parts of the tumor.[11] This leads to misclassification in less than 1% of patients.[11,12] Dopierala *et al.*[22] analyzed 32 UMs by multiplex ligation dependent probe amplification for different parts of the tumor and reported heterogeneity of chromosome 3 in 47% of cases, not leading to misclassification when compared with the whole tumor. The MLPA technique provides a relative quantification of monosomy 3 (and multiple other chromosomal regions) and cells with disomy 3 in the different tumor regions may dilute the obtained results. Fluorescence in situ hybridization, on the other hand, enables absolute quantification of monosomy 3 in single tumor cells. The problem of sampling error and misclassification is thought to be less important if larger enucleation specimens are used, as these are more representative of the tumor than biopsies.[21,23] In this study, larger enucleation specimens were used from the patient tumors, minimizing the risk of misclassification.

Single nucleotide polymorphism array is a recent molecular genetic technique based on a series of DNA segments orderly arranged on a chip to which fluorescently labeled DNA can be hybridized. With this technique, rapid assessment of copy number alterations as well as zygosity changes on a genome-wide level with a high resolution is possible. MLPA allows for copy number analysis of up to 50 chromosomal regions in one experiment and is also less labor-intense than the FISH technique. Nevertheless, an important advantage of the FISH technique is that absolute copy numbers can be assessed and low mosaic cases (alterations in low percentage of cells) can be detected, which is more difficult with SNP-array and MLPA. This study demonstrated that even patients with low percentage

aneuploidy of chromosomes 3 and 8q, who are at risk for developing metastasis, can be identified by FISH with absolute quantification of additional copies of chromosome 8q as well.

This study is, based on current literature and data, the first to use incrementally increasing FISH counts of chromosome 3 losses and 8q gains, and evaluate its impact on disease-free survival. In total, 220 patients were studied and analyzed by FISH, providing first steps toward a more individualized prognosis for UM patients. Future studies are needed in order to obtain more cases with low and intermediate percentages of chromosome 3 loss and chromosome 8q gain and enable an even more reliable comparison of these groups. There is a bias toward the larger tumors, since only enucleated eyes were included in this study. In the future, in-vivo biopsy of UMs treated by eye-sparing techniques may provide new information on the distribution of chromosome 3 and 8q alterations in small and medium-sized tumors. The importance of chromosome 3 alterations in UM was recently further demonstrated by Harbour *et al*.,[24] who reported on frequent somatic and one germline *BAP1*-mutation, located on chromosome 3p21.1, in class 2 metastasizing melanomas. In a previous study from this group, monosomy 3 was detected in four out of five class 2 tumors.[25] It would be interesting to assess whether patients with monosomy 3 tumors from this present study have a mutant *BAP1*-gene on the remaining allele as well. If so, then it is worthwhile to determine whether *BAP1* mutations have a better predictive value than monosomy 3.

In conclusion, patients having tumors with a high percentage of monosomy 3 have a slightly worse 4-year overall survival probability than patients with monosomy 3 tumors in general. The patients with an increased number of additional copies of chromosome 8q in their tumor are at risk of early metastasis. Since patients with a high percentage monosomy 3, intermediate or high percentage gain of chromosome 8q, and additional copies of 8q in their tumor cells have a high risk of early metastasis, this group could be eligible for adjuvant treatment preventing the development of metastasis. Therefore, results of adjuvant therapies may be observed much earlier within this group than with classic long-term studies.

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CHAPTER 6

METASTATIC DISEASE IN UVEAL MELANOMA: IMPORTANCE OF A GENETIC PROFILE?

Jackelien van Beek
Anna Koopmans
Jolanda Vaarwater
Robert Verdijk
Annelies de Klein
Nicole Naus
Emine Kiliç

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ABSTRACT

Mutation of *SF3B1* has been identified in low-grade uveal melanoma with a good prognosis. In this study, we compare chromosomal aberrations and gene mutations between a primary uveal melanoma and its multiple hepatic and peripancreatic metastases. DNA was isolated from a large primary uveal melanoma after fractionated stereotactic radiotherapy and three distinct metastases (two liver samples and one peripancreatic lymph node) to perform single nucleotide polymorphism array and fluorescent *in situ* hybridization. We analyzed mutations in uveal melanoma target genes *BAP1*, *GNAQ*, *GNA11*, *SF3B1*, and *EIF1AX*. The primary tumor showed no abnormalities in chromosome 3, whereas metastases showed deletion of at least 3q12.1-q24 and the *BAP1* gene was not mutated. All samples indicated the following consistent chromosomal aberrations: loss of 1p, gain of 6p, and gain of 8q. Subsequently, heterozygous *SF3B1* and heterozygous *GNA11* mutations were observed. The metastases showed more genetic aberrations than the primary tumor and may therefore represent the genetic status of the tumor before irradiation, whereas the current primary tumor shows presumably irradiation artifacts. An early occurring mutation in *GNA11* was observed in all samples. The *SF3B1* mutation seems to predispose for late metastatic disease in the absence of a *BAP1* mutation.

INTRODUCTION

Uveal melanoma (UM) are known to spread to the liver and less frequently to the lungs, bones, skin, and brain.[1] Metastatic disease occurs in half of the UM patients and predominantly in those with monosomy 3 tumors. Recent studies have identified *BAP1* mutations in metastatic UM.[2,3] Mutations of *SF3B1* at codon 625 and *EIF1AX* have been identified in low-grade UM with a good prognosis.[4,5] *GNAQ* and *GNA11* mutations occur in the majority of UM patients and are not associated with survival.[6]

We present a patient with an UM and late occurring metastases in the liver and peripancreatic lymph node. We performed single nucleotide polymorphism (SNP) array analysis and fluorescent *in situ* hybridisation (FISH) on the primary tumour and metastatic tissue. Additionally, we analysed UM target genes *BAP1, GNAQ, GNA11, SF3B1* and *EIF1AX*.

CASE REPORT

In March 2001, a 45-year-old man presented with a decreased vision in his right eye (20/60) and normal vision in the left eye (20/20), with normal intraocular pressure. Indirect ophthalmoscopy and ultrasonography (US) of the right eye showed a dome-shaped UM located temporal superior (**Figure 1A**) with a low to medium internal reflectivity, choroidal excavation, and a largest basal diameter of 15.2 × 14.6 mm and a thickness of 7.8 mm.

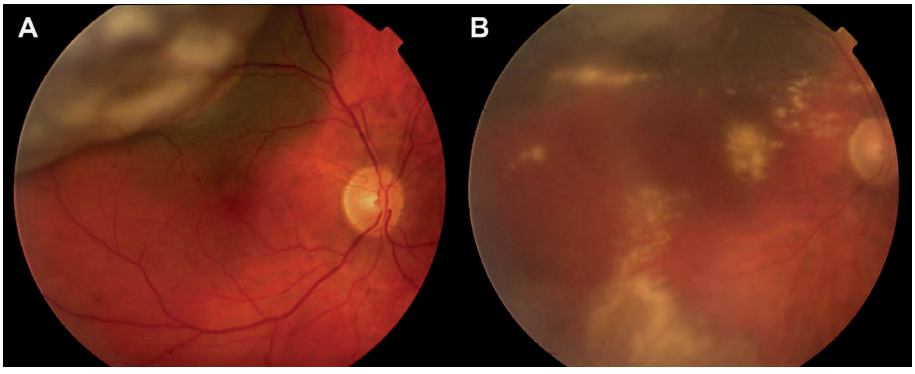


Figure 1. A dome-shaped uveal melanoma of the right eye in 2001 (**A**). In 2005, ischemic irradiation retinopathy developed after fractionated stereotactic radiation therapy (**B**).

The γ-glutamyl transpeptidase was high, compatible with a pre-existent alcohol abuse. Metastatic workup, including abdominal US, showed no abnormalities. Despite the large tumor, the patient opted to undergo fractionated stereotactic radiotherapy with a total dose of 50 Gy, and according to protocol, a MRI and computed tomographic scan was performed for fractionated stereotactic radiotherapy treatment planning. In 2004, the patient developed angle-closure glaucoma because of lens swelling and underwent cataract surgery.

In January 2005, the tumor had regressed and a thickness of 2.9 mm was measured on US. Eventually, he developed therapy-resistant neovascular glaucoma because of ischemic retinopathy (**Figure 1B**) and the eye was enucleated. Histopathologic analysis showed a mixed-cell malignant melanoma without ciliary body and scleral invasion, absence of extracellular matrix patterns, and mitotic figures in 4/8 mm^2 (equivalent to 50 high-power fields). The center and base of the tumor were necrotic without any obvious signs of inflammation. During follow-up visits, only γ-glutamyl transpeptidase values were elevated between 65 and 150 U/l. In the meantime, the patient requested referral to his primary ophthalmologist.

In October 2009, the patient presented with abdominal pain and the computed tomographic scan showed several liver metastases and a peripancreatic lymph node metastasis of 5.9 mm. The patient died one month later and metastatic tissue became available with patients' consent. Autopsy revealed that 70% of the liver was infiltrated with UM metastases. As in the primary tumor, mixed-cells were found in the liver metastasis and in the peripancreatic lymph node.

METHODS

Fresh tumor tissue was harvested and DNA was isolated as described previously.[6,7] Autopsy was performed within 4 days after death and metastatic tissue of liver and peripancreatic lymph node was fresh frozen. To determine copy number variations, the Illumina Human CytoSNP12 Beadchip (Illumina, San Diego, California, USA) was used and data were analyzed using Nexus version 6 (Nexus BioDiscovery, El Segundo, California, USA). Chromosomal abnormalities were validated with FISH hybridization analysis on directly fixed tumor cells using centromeric or locus-specific probes: 1p36.33, 1p12, 1q21.1, 3p13, centromere 3, 3q11.2, 3q13.3, 3q22, 3q25.1, 3q26, 6p22, 6q21, 8p21.3, centromere 8, 8q22, and 22q11.22. Chromosome region 5q21.1 was used as a control. Exons 4 and 5 of *GNAQ* and *GNA11*, and the entire *BAP1* gene were sequenced as described previously.[3,6] Exon 14 of *SF3B1* (including hotspot R625), and exons 1 and 2 of *EIF1AX* were analyzed with Sanger sequencing (protocols available upon request). In addition, the expression of *BAP1* protein was assessed by immunohistochemistry as described by Koopmans *et al.*.[3]

RESULTS AND DISCUSSION

Chromosomal alterations and gene mutations such as (incomplete and complete) loss of 1p, gain of 6p, gain of 8q, a heterozygous *GNA11* mutation, and a heterozygous mutation in *SF3B1* were observed in all samples. The metastases contained more chromosomal aberrations compared with the primary tumor, such as gain of 1q, loss of region 3q11.2-3q24, and loss of 6q (**Figure 2** and **Figure 3**). All samples showed no *BAP1* mutation and a positive *BAP1* expression.

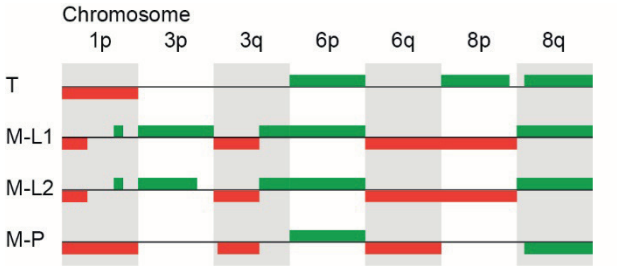
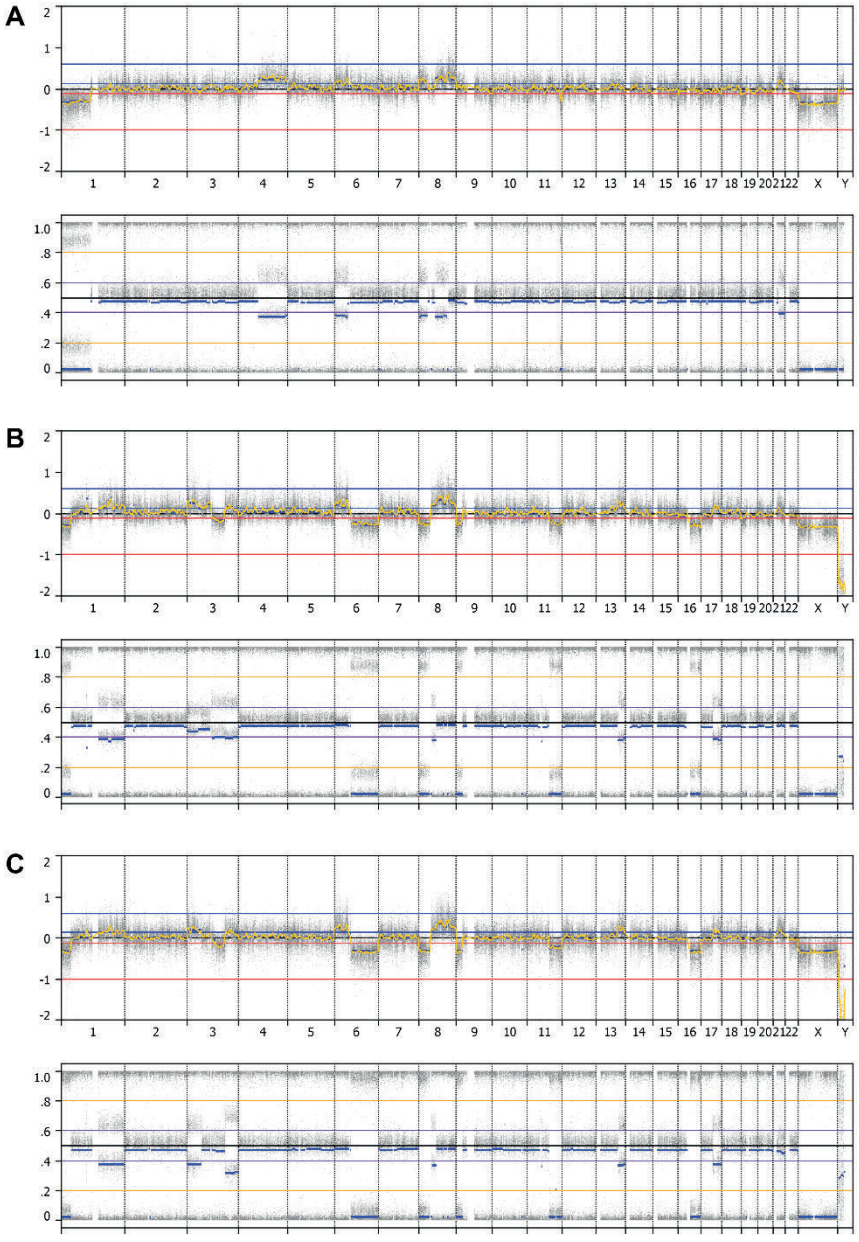


Figure 2. A schematic summary of the chromosomal abnormalities detected by single genome nucleotide polymorphism array analyses in the primary tumor (T), liver metastasis 1 (M-L1), liver metastasis 2 (M-L2), and the peripancreatic lymph node (M-P). The red and green bars represent loss and gain, respectively.

The primary tumor as well as metastatic tissue harbored a mutation in exon 5 of the *GNA11* gene. Most of the UM contain mutations in *GNAQ* and *GNA11* and these mutations are considered to occur early in UM development.[8] Estimates of tumor doubling times range between 30 and 80 days and support the hypothesis that UM with the propensity to metastasize do this when they are small, and before detection and treatment of the primary tumor.[9]

Even though several studies on UM metastases describe that most metastases reflect the primary tumor, the patient's primary tumor was irradiated and shows differences from its metastases (**Figure 2** and **Figure 3**).[10] In metastatic samples, we found a gain of 8q and a partial loss of chromosome 3q. In the pathogenesis of UM, monosomy 3 is considered to be an early event and gain of 8q a secondary hit.[11] A large tumor diameter, such as the primary tumor, is associated with a high percentage gain of chromosome 8q.[12] Trolet *et al.*[10] also observed a higher level of 8q gain in monosomy 3 metastatic tumors compared with monosomy 3 nonmetastatic UM. As for abnormalities of chromosome 6, these have been associated with a good prognosis, whereas loss of chromosome 6q is correlated with decreased survival and has been considered to be a secondary event.[11,13]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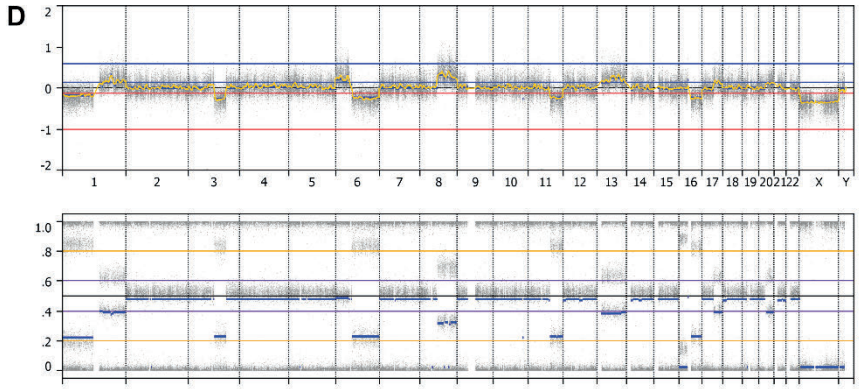

Figure 3. Whole-genome single nucleotide polymorphism array analyses in the primary tumor (**A**), liver metastasis 1 (**B**), liver metastasis 2 (**C**) and the peripancreatic lymph node (**D**). Each panel includes plot of log R ratio (upper panel) and B allele frequency (lower panel).

The metastases indicated more chromosomal abnormalities compared with the primary tumor. With radiation applied to the primary tumor, cell killing is assumed to occur through DNA cluster damage. This leads to chromosome aberrations and eventually to clonogenic inactivation.[14] Therefore, early mutations, such as *GNA11*, were present in all cells. *BAP1* mutations, which are assumed to occur later and predispose for metastatic disease, were absent in the primary tumor, and also in the metastases. *BAP1* expression was found to be positive. Monosomy 3 and *BAP1* mutation are associated with a poor prognosis. However, a subset of UM with a monosomy 3 do not have a *BAP1* mutation.[2,3] If patients develop metastases, they occur much later than metastases in the patients with a *BAP1* mutated tumor.[3] *SF3B1* mutations have been associated with good prognostic parameters.[4,5] We observed an *SF3B1* mutation in the primary tumor and metastases in the absence of a *BAP1* mutation. This implies that a *SF3B1* mutation does not protect from metastases, and that there are different mechanisms involved in developing metastatic disease in UM.

CONCLUSION

We describe an UM with its corresponding metastases that occurred many years after primary treatment. Besides loss of 1p and gain of 8q, a *SF3B1* mutation seems to predispose for late metastatic disease in the absence of a *BAP1* mutation.

ACKNOWLEDGEMENTS

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PART II

TREATMENT OUTCOME OF UVEAL MELANOMA AND QUALITY OF LIFE IN RELATION TO TREATMENT

CHAPTER 7

FRACTIONATED STEREOTACTIC RADIOTHERAPY FOR UVEAL MELANOMA: LONG-TERM OUTCOME AND CONTROL RATES

Jackelien van Beek
Caroline van Rij
Sara Baart
Serdar Yavuzyigitoglu
Michael Bergmann
Dion Paridaens
Nicole Naus
Emine Kiliç

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ABSTRACT

Purpose. The aim of our study is to evaluate local tumour control rates, radiation side effects, visual preservation and disease-free survival (DFS) of uveal melanoma (UM) patients treated with fractionated stereotactic radiotherapy (fSRT).

Methods. A retrospective study of UM patients, who were treated with fSRT (n = 189) was performed by the Rotterdam Ocular Melanoma Study group (ROMS), The Netherlands, between 1999-2014 with a follow-up of at least five years.

Results. The 1, 3, 5, 10 and 15-years local tumour control rates were: 99.4%, 92.8%, 92.2%, 89.3% and 89.3%, respectively. Cataract (67.8%) was the most common side effect of fSRT followed by retinopathy (35.1%), maculopathy (23.8%), vitreous haemorrhage (20.1%), neovascular glaucoma (NVG) (20.0%) and optic neuropathy (12.4%). Patients with anterior located UMs developed cataract more frequently ($p = 0.047$, multivariable analysis). By multivariable analysis significant factors for secondary enucleation were tumour recurrence ($p < 0.001$) and NVG ($p < 0.001$). In multivariable analysis, risk factors for a worse DFS were larger UM ($p = 0.024$) and tumours with subretinal fluid at baseline ($p = 0.038$). The 5-years DFS was 77.0% and the best corrected visual acuity decreased significantly after treatment. After 5 years, 22.0% of patients and after 10 years 17.6% of patients had a visual acuity of ≤ 0.3 logMAR.

Conclusions. Fractionated stereotactic radiotherapy is a good treatment option for small, medium and large sized tumours with 5-years local tumour control of 92.2%. After 5 years, 22.0% of the patients had a good vision. Independently of tumour location the visual acuity decreased significantly after treatment. Overall, the 5-years DFS was 77.0%.

INTRODUCTION

Uveal melanoma (UM) are rare ocular tumours, but related with significant morbidity and mortality. The treatment of UM depends on the size and the location of the tumour, the secondary effects of the tumour on the eye, the status of the fellow eye and patients' choice. The last decades eye-preserving therapies, have proven to be equally effective regarding overall patient survival and metastasis free survival compared to enucleation.

Fractionated stereotactic radiotherapy (fSRT) is a treatment for mostly small- and medium-sized UM and some larger UM up to approximately 12 mm in thickness and a diameter smaller than 16 mm. Other radiotherapeutic treatment options for UM of these size are proton beam radiotherapy and brachytherapy. One advantage of fSRT is that fSRT requires no surgical procedures to determine the tumour localisation and dimensions. Reported side effects are similar to those of brachytherapy and proton beam radiotherapy and can lead to visual impairment and secondary enucleation (3-16%).[1-4] Radiogenic side effects are cataract, retinopathy, maculopathy, neovascular glaucoma, vitreous haemorrhage and optic neuropathy.[5-9]

The aim of this study was to evaluate the value of fSRT for treatment of UM. Since there are overlapping treatment options depending on the tumour size and location, it is important to know the potential radiogenic side effects and how to treat them. Awareness of the risk of side effects can influence the choice of treatment. Since some side effects develop over the years, we only analysed our treated patients with a follow-up of at least five years. For this subset, we report local tumour control rate, (late) radiogenic side effects (i.e., secondary glaucoma, cataract, vitreous haemorrhage, radiation inducted optic neuropathy, radiation induced maculopathy and retinopathy), visual preservation and disease-free survival.

MATERIALS AND METHODS

Patients

A retrospective study by the Rotterdam Ocular Melanoma Study group (ROMS), was performed in 189 patients with choroidal and/or ciliary body UM treated with fSRT at the Radiation Oncology department of the Erasmus MC, Rotterdam, The Netherlands, between 1999 and 2014. We excluded iris melanomas as well as posterior UMs that were larger than 12 mm in thickness and/or had a larger diameter than 16 mm. The latest follow-up date was January first 2020. All patients had a follow-up of at least 5 years. The local medical ethical committee approved the retrospective data-analysis for the long-term effects and treatment outcome.

The patients were treated in a standardized way and were informed on the treatment and follow-up protocol for this treatment. The study was performed according to guidelines of the Declaration of Helsinki.

At time of diagnosis, patients were examined by an ophthalmologist with expertise in ocular oncology. Fundus photography and B-scan ultrasonography were used for follow-up. If necessary, ancillary tests were used, such as fluorescent angiography, indocyanine green angiography and A-scan ultrasonography. All patients underwent full systemic examination and staging evaluation by a specialised oncologist from our institution. All UM patients received primary radiation and were treated by a specialised radiation oncologist. All clinical and follow-up data was collected and processed in a homemade database application based on Filemaker 16 (FileMaker Inc, Santa Clara, California, United States). UM were categorized according to the Tumour, Nodes, Metastasis (TNM) Classification; the Tumour (T) category of the 8th edition of the American Joint Committee on Cancer (AJCC) staging system of posterior uveal melanoma.[10] The tumour (T) is given a classification of T1 to T4 based on its width (largest basal diameter) and height (thickness).

Our fSRT treatment protocol has been described previously. The stereotactic radiation dose is given in 5 fractions of 10 Gray (total 50 Gray), at the 80% isodose over five consecutive days.[5,11]

The following side effect endpoints were counted: neovascular glaucoma, cataract, vitreous haemorrhage (VH), optic neuropathy, maculopathy with or without cystoid macular edema, retinopathy, subretinal fluid, local recurrence, secondary enucleation and the visual acuity. Neovascular glaucoma can present through either a secondary open-angle or secondary closed-angle mechanism depending on the extent of neovascularization. Cataract was defined as lens opacities that developed after treatment and patients with cataract and/or phacoemulsification before treatment were excluded. Vitreous haemorrhage was counted as a side effect when a haemorrhage occurred after treatment. Patients with a VH before treatment were excluded. Optic neuropathy was defined as visual loss caused by collateral optic nerve damage and diminished colour vision (using the test of Ishihara) with or without a relative afferent pupillary defect. Presence of maculopathy was defined as haemorrhages, hard exudates and edema in the macula. Cystoid macular edema (CME) was noted clinically on fundus examination, on optical coherence tomography (OCT) or on fluorescent angiography when available. CME was counted as maculopathy. We excluded cystoid macular edema after cataract extraction. After 2007 intravitreal injections of anti-vascular endothelial growth factor (VEGF) became available in our clinic. Retinopathy was marked by damage to retinal blood vessels with bleedings, swelling of the retina, or abnormal growth of new blood vessels. Subretinal fluid was counted

as a side effect, when it developed after treatment. Presence of subretinal fluid before treatment was excluded. Local recurrence of UM was determined clinically, with ultrasonography and by comparing fundus photos. We noted the cause of enucleation. From the medical history we had recorded hypertension and/or diabetes mellitus. The visual acuity was measured with Snellen chart and converted to a logMAR score. We defined mild distance visual impairment as a visual acuity worse than 6/12 (0.3 logMAR) to 6/18 (0.5 logMAR), moderate vision impairment corresponds with a visual acuity worse than 6/18 (0.5 logMAR) to 6/60 (1.0 logMAR) and severe vision impairment corresponds with a visual acuity worse than 6/60 (1.0 logMAR) to 3/60 (1.3 logMAR). Blindness corresponds with a visual acuity worse than 3/60 (1.3 logMAR). Local tumour control was defined as tumour growth after initial treatment; secondary enucleation due to tumour growth; additional treatment due to tumour growth or regrowth after initial regression of the tumour. Disease-free survival (DFS) included time from treatment until metastases or death were diagnosed.

Statistical analyses

General patient and tumour characteristics, and side effects after treatment were analysed using Mann-Whitney U test and Chi-square statistics. Endpoints are defined as the time between treatment and a side effect or in case of survival as the time between treatment and metastases or death. Follow-up duration, used as the time variable, was measured from the date of treatment to the latest visit. The risk of a side effect caused by a tumour characteristic was analyzed by applying Cox proportional hazard models to calculate hazard ratios (HR) with corresponding 95% confidence intervals (CI). Unless explicitly mentioned otherwise, we used univariable analyses for the side effects. All side effects were analysed. Multivariable models were estimated by including variables that were significant in the univariable analyses and, if appropriate, additional covariates based on clinical expertise, while adhering to the rule of thumb of ten events per variable. If there were no significant variables in the univariable analysis, we added age at diagnosis and sex to the multivariable model.

For the best corrected visual acuity (BCVA) Wilcoxon signed-rank tests were used to compare the BCVA of the five timepoints. We adjusted the significance level for BCVA to the p-value ≤ 0.01 because of multiple testing.

Cox's proportional hazards models were performed to assess statistical significance between the curves. Finally, the actuarial rates were calculated at 1, 3, 5, and 10 years of follow-up. We considered a p-value ≤ 0.05 as statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 22.0 for Windows (SPSS inc., Chicago, IL, USA).

RESULTS

Patient and tumour characteristics

Our population included 189 UM patients treated with fSRT with at least a follow-up of five years (median 92.9 months, IQR: 55.4-134.7 months). All patients and tumour characteristics are presented in **Table 1.** One T4 tumour was included and found to be suitable for fSRT, due to a thickness of 9.2 mm and with an oval shape (basal diameter of 12.8 x 18.5 mm). Another factor influencing the choice of treatment was the state of the other eye, as the treated eye had the best visual potential due to an amblyopic fellow eye (visual acuity of 0.70 logMAR/ 0.2 Snellen). Four patients (2.1%) were lost to follow-up for information on side effects with a median of 104.5 months disease-free survival months (IQR: 39.2-149.0 months). One patient died due to other cause, while having metastases of UM. Associations with age, sex and visual acuity at baseline were examined for all tumour characteristics (**Table 1**). Only a significant association was observed between the T category of the TNM classification and sex (p = 0.048, Pearson Chi-square test). Male patients (49.5%) had the most T3/T4 category UM, while female patients (52.2%) had the most T2 category UM. The side effects after fSRT are shown in **Table 2**.

Table 1. General characteristics of the total population at baseline and after treatment with fractionated stereotactic radiotherapy (fSRT) for uveal melanoma

Patient characteristics	**Population fSRT (N=189) N (%)**	**Median and IQR *in mm or months***
Age (mean± SD) *in years*	62.1 ± 11.1 range (28.1-84.0)	
Sex (N=189)		
Female	92 (48.7)	
Male	97 (51.3)	
Affected eye (N=189)		
OD	89 (47.1)	
OS	100 (52.9)	
Tumour characteristics		
Shape (N=189)		
Dome	145 (76.7)	
Mushroom	38 (20.1)	
Diffuse	3 (1.6)	
Unknown	3 (1.6)	
Tumour Pigmentation, Yes (N=187)	165 (88.2)	
Orange Pigment, Yes (N=177)	75 (42.4)	
Vitreous haemorrhage pre-treatment (N=188)	11 (5.9)	
Drusen, Yes (N=180)	56 (31.1)	

Table 1. *(continued)*

Patient characteristics	Population fSRT (N=189) N (%)	Median and IQR *in mm or months*
Tumour characteristics		
Subretinal fluid pre-treatment (N=186)		
Grade 1	57 (30.7)	
Grade 2	46 (24.7)	
Grade 3	13 (7.0)	
TNM class, T category (N=189)		
1	28 (14.8)	
2	83 (43.9)	
3	77 (40.7)	
4	1 (0.5)	
Margin to fovea ≤ 3 mm (N=189)	102 (54.0)	
Margin to fovea *in mm*		Median: 3.0 (IQR: 1.0-6.0)
Margin to optic disc ≤ 3 mm (N=189)	94 (49.7)	
Margin to optic disc *in mm*		Median: 3.0 (IQR: 2.0-5.1)
Metastases		
Metastases and development of metastases (N=189)	54 (28.6)	
Disease-free survival overall (N=189) *in months*		Median: 91.6 (IQR: 41.9-132.5)
Disease-free survival in metastases-group (N=54) *in months*		Median: 30.0 (IQR: 19.3-51.3)
Status at the end of the study		
Alive without metastases, Disease-free survival *in months*	88 (46.6)	Median: 115.6 (IQR: 84.4-164.0)
Alive with known metastases	3 (1.6)	Median: 163.7
Death through UM, Disease-free survival *in months*	49 (25.9)	Median: 28.9 (IQR: 20.0-48.0)
Death other cause, Disease-free survival including 1 patient with metastases *in months*	33 (17.5)	Median: 89.1 (IQR: 41.1-104.5)
Lost to follow-up, Disease-free survival *in months*	4 (2.1)	Median: 60.8 (IQR: 16.1- 95.2)
Medical history pre-treatment		
Diabetes mellitus (N=189)	16 (8.5)	
Hypertension (N=189)	58 (30.7)	

SD = standard deviation; TNM = primary tumor (T), regional lymph nodes (N), distant metastases (M); IQR = interquartile range

Local tumour control

In the study population (n = 185) local tumour control was achieved in 91.4% (**Figure 1**). Some patients had a follow-up of 19 years. The 1, 3, 5, 10 and 15-years cumulative local tumour control rates were: 99.4%, 92.8%, 92.2%, 89.3% and 89.3% respectively. Of the 185 patients only 16 UM recurred with a median time of 19.8 months (IQR: 15.3-37.1 months). Of the sixteen (8.7%) tumour recurrences (**Table 3**): ten eyes (5.4 %) were enucleated due to tumour progression and six (3.2%) tumours received only trans pupillary thermotherapy (TTT). Two out of the ten enucleated patients due to tumour progression received TTT before enucleation. Two additional enucleations occurred due to later developed neovascular glaucoma (**Table 3**). After additional treatment no recurrence was observed. Twelve tumours with recurrences were treated before 2007 with a median time to recurrence of 21.4 months versus tumours treated after 2007 with a median time to recurrence of 17.6 months (p=0.055). Tumours with subretinal fluid at baseline were more likely to recur (HR: 4.81, 95% CI 1.08-21.31, Cox proportional hazard models), while larger UM did not recur more. Eyes with tumour recurrence were significantly more enucleated (HR: 14.35, 95% CI 4.62-44.50). This applies to univariable analysis and multivariable analysis with enucleation and subretinal fluid at baseline in the model; significant more eyes were enucleated ($p < 0.001$; Supplementary **Table 4a**).

Table 2. Side effects after fractionated stereotactic radiotherapy (fSRT) for uveal melanoma

Side effects	**Study population fSRT n=185 (%)**	**Median and IQR *in months***
Neovascular glaucoma, (n=185)	37 (20.0)	Median: 21.1 (IQR: 14.3-42.3)
Cataract, (n=149) excluded due to pre-treatment cataract (n=36)	101 (67.8)	Median: 24.3 (IQR: 8.3-42.5)
Vitreous haemorrhage (VH), (n=174) excluded due to pre-treatment VH (n=11)	35 (20.1)	Median: 24.8 (IQR: 10.5-41.6)
Optic neuropathy, (n=185)	23 (12.4)	Median: 23.6 (IQR: 14.0-36.9)
Maculopathy, (n=185)	44 (23.8)	Median: 29.3 (IQR:11.3-54.0)
Retinopathy, (n=185)	65 (35.1)	Median: 26.3 (IQR: 16.7-48.2)
Subretinal fluid (SRF) (n=151) excluded due to pre-treatment SRF (n=34)	10 (6.6)	Median: 12.1 (IQR: 2.1-96.1)

IQR = interquartile range

Ocular side effects

Radiation induced side effects after fSRT vary from very mild to severe. Thirty-six patients (19.5%) did not develop any radiogenic side effects after a median follow-up of 39.3 months (IQR: 21.3-86.1 months). Of these 36 patients, 8 patients were still alive and had no metastases at the end of the study after a median follow-up of 93.4 months (IQR: 77.5-139.0 months). The following late side effects after fSRT (**Table 2**) were observed:

Neovascular glaucoma (NVG): Of the 37 patients, who developed NVG, significantly more patients had larger UM (p = 0.030). T3 and T4 tumours were 3.8 times more likely to develop NVG compared to T1 tumours alone (HR: 3.82, 95% CI 1.14-12.84). NVG was controlled with medication and/or laser treatment in 22 eyes and treated with anti-VEGF intravitreal injections in 13 eyes. Often combinations of those treatments were given. Sixteen eyes were eventually enucleated due to uncontrollable NVG, resulting in a blind eye. Eyes with NVG had significantly (HR: 7.15, 95% CI 3.73-13.71; p < 0.001) more enucleations. After multivariable analysis with the following variables: age at diagnosis, enucleation and TNM classification; this effect between NVG and enucleation remained significant (p < 0.001; Supplementary **Table 4b**). The median period for an enucleation after NVG was 31.5 months (IQR: 16.3-69.5 months).

Cataract: Thirty-six (19.5%) of the 185 patients were excluded, because they already had pre-treatment cataract. In total 101 of the 149 patients (67.8%) developed cataract after fSRT. The degree and associated symptoms of the cataract varied. Therefore, of the patients with cataract (n =101), only 42 patients (41.6%) were treated with phacoemulsification after a median period of 21.7 months (IQR: 13.7-48.1 months). Larger UM within categories T3/T4 (versus category T1 UM (HR: 2.35, 95% CI 1.23-4.50)) and tumours further from the fovea developed significantly more cataract than tumours closer to the fovea (p=0.025; HR: 1.09, 95% 1.01-1.17). Eyes with T2 tumours did not develop more cataract. We estimated a multivariable model with the following covariates: tumour T(NM) classification, margin to the fovea, margin to the disc, age at diagnosis and sex. By multivariable analysis, only UM further from the fovea developed significantly more cataract (p = 0.047; Supplementary **Table 4c).**

Table 3. Clinical characteristics of 16 uveal melanoma patients with tumour recurrence

N	Sex	Age (years)	TNM class, T category	Margin to fovea ≤ 3mm or > 3 mm	Margin to optic disc ≤ 3 mm or > 3 mm	Year of treatment	Timo to meta (months)	DFS (months)	Time to tumour recurrence (months)	Time to TTT (months)	Time to secondary enucleation (months)	Time to NVG (months)	Cause of enucleation
1	F	65	1	< 3 (1 mm)	< 3 (0 mm)	2001	-	217.2	83.7	-	84.8	-	Tumour progression
2	M	61	3	≤ 3 (3 mm)	< 3 (0 mm)	2003	-	193.5	16.4	16.4 20.4 23.4	42.6	26.7	Tumour progression
3	M	44	3	< 3 (0 mm)	< 3 (0 mm)	2002	-	198.7	23.6	23.6	63.4	-	Tumour progression
4	M	56	2	> 3 (5 mm)	> 3 (5 mm)	2003	-	133.1	81.8		82.3	-	Tumour progression
5	M	79	3	> 3 (8 mm)	> 3 (8 mm)	2003	25.4	25.4	15.3	-	15.3	-	Tumour progression
6	M	83	2	≤ 3 (3 mm)	≤ 3 (3 mm)	2006	35.7	35.7	14.0	-	14.0	-	Tumour progression
7	F	67	2	< 3 (0 mm)	< 3 (1 mm)	2006	-	158.8	40.4	-	40.4	-	Tumour progression
8	M	66	3	> 3 (8 mm)	> 3 (6 mm)	2010	-	103.0	79.9	-	80.1	42.8	Tumour progression
9	M	57	3	> 3 (4 mm)	> 3 (4 mm)	2012	32.8	32.8	19.8	-	21.2	-	Tumour progression
10	F	54	3	≤ 3 (3 mm)	> 3 (4 mm)	2012	27.9	27.9	15.4	-	15.8	-	Tumour progression
11	M	50	2	< 3 (1 mm)	≤ 3 (3 mm)	2001	-	202.1	15.3	15.3	44.5	40.7	NVG

N	Sex	Age (years)	TNM class, T category	Margin to fovea ≤ 3mm or > 3 mm	Margin to optic disc ≤ 3 mm or > 3 mm	Year of treatment	Timo to meta (months)	DFS (months)	Time to tumour recurrence (months)	Time to TTT (months)	Time to secondary enucleation (months)	Time to NVG (months)	Cause of enucleation
12	M	77	3	≤ 3 (3 mm)	> 3 (6 mm)	2003	-	136.7	22.8	22.8	74.5	70.3	NVG
13	F	66	2	> 3 (6 mm)	≤ 3 (3 mm)	2001	32.4	32.4	27.2	27.2	-	-	-
14	M	53	2	< 3 (0 mm)	< 3 (1 mm)	2002	-	106.9	19.9	19.9	-	-	-
15	M	48	1	< 3 (1 mm)	< 3 (2 mm)	2002	-	188.8	9.0	9.0	-	-	-
16	F	51	1	< 3 (0 mm)	< 3 (1 mm)	2011	-	93.7	13.2	13.2	-	-	-

TNM = primary tumor (T), regional lymph nodes (N), distant metastases (M); DFS = disease-free survival; TTT = trans pupillary thermotherapy; NVG = neovascular glaucoma

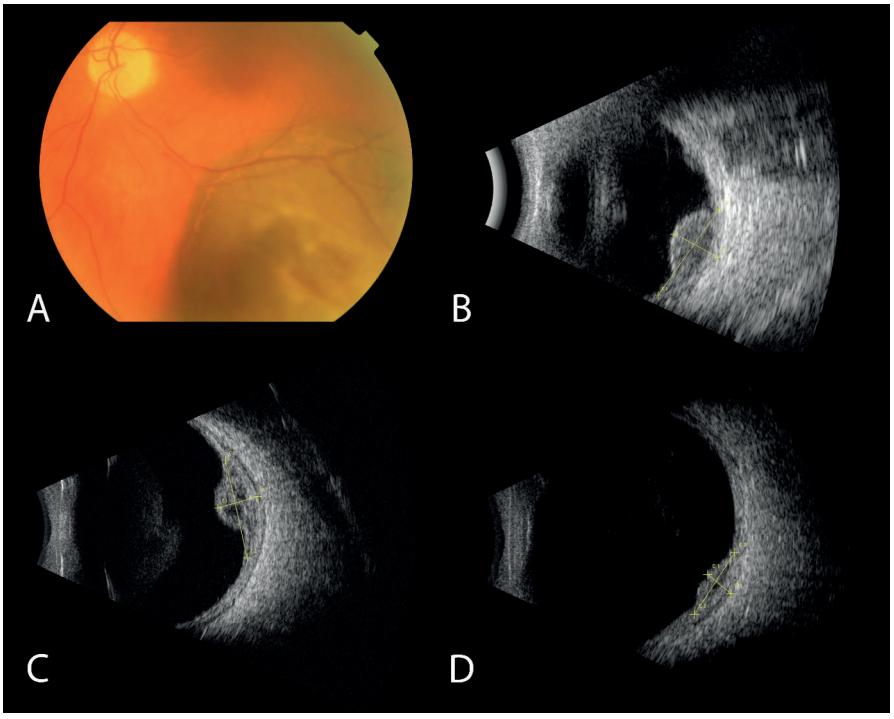

Figure 1. A 66-year-old woman with an uveal melanoma was treated with fractionated stereotactic radiotherapy. At presentation, a T2 tumour is visible temporal inferior of the macula of the right eye (**A**) and with a tumour thickness of 5.7 mm and a largest basal diameter of 12.1 mm on ultrasound (**B**). After treatment, ultrasound images showed tumour regression: with a thickness of 4.4 mm after one year (**C**) and a thickness of 2.7 mm after two years (**D**). Yellow lines show the points of the tumour measurements.

Vitreous haemorrhage (VH): In total 35 (20.1%) of the 174 patients developed a VH. We excluded 11 patients with a VH at baseline. Diabetes mellitus did not influence the occurrence of VH. Patients with hypertension developed significantly less VH (p = 0.026), while patients with a lower age (p = 0.006, HR: 0.96, 95% CI 0.94-0.99) developed more VH. In multivariable analyses with age at diagnosis and sex added to the model; hypertension was not a significant protective factor, while age at diagnosis remained a significant factor for the development of VH (p = 0.031; Supplementary **Table 4d**). The mean age at diagnosis in the group with VH was 57 years and in the group without VH was 63 years.

Optic neuropathy: Patients with a lower age (HR: 0.96, 95% CI 0.93-1.00) and tumours with less pigment (HR: 0.29, 95% CI 0.12-0.70) developed more optic neuropathy. Additionally, tumours closer to the optic disc were significantly associated with optic neuropathy (HR: 0.82, 95% CI 0.68-1.00). In multivariable analysis with age

at diagnosis, pigmentation of the UM and margin to the disc added to the model: only UM with less pigment developed significantly more optic neuropathy (p = 0.008; Supplementary **Table 4e**).

Radiation maculopathy: Of the 44 eyes with maculopathy, 35 eyes had cystoid macular edema and nine eyes developed an ischemic maculopathy without edema. UM treated after the year 2007 developed more and significantly earlier maculopathy (median 29.3 months) than before 2007 (p = 0.003) (median 37.4 months). Tumours closer by the optic nerve or to the fovea were not associated with maculopathy. After multivariable analysis with age at diagnosis and sex added to the model: treatment after 2007 remained a significant factor for the development of maculopathy (p = 0.003; Supplementary **Table 4f**).

Radiation retinopathy: Younger patients (HR: 0.97, 95% CI 0.95-0.99) developed more radiation retinopathy. The mean age at diagnosis in the group with retinopathy was 59 years and in the group without retinopathy was 64 years.
Only T1 tumours were associated with retinopathy in univariable analysis (HR: 1.95, 95% CI 1.00-3.78) compared to other T category tumours. A multivariable model with the covariates: age at diagnosis, TNM classification, margin to the fovea and to the disc was estimated. In this model lower age at diagnosis (p = 0.007) and TNM classification, T1 tumours compared to T3/T4 UM were significantly independently associated with radiation retinopathy (p = 0.021; Supplementary **Table 4g**). We found no evidence that diabetes mellitus or hypertension influenced the occurrence of retinopathy, maculopathy or neovascular glaucoma.

Subretinal fluid (SRF): Of the 116 UM with SRF at baseline (**Table 1**), in 82 of these UM the fluid resolved after treatment. SRF remained in 34 UM and these 34 patients were therefore excluded for analyses about the posttreatment side effect of SRF (**Table 2**). Only 10 (6.6%) UM of the remaining 151 UM developed SRF. The development of SRF was not influenced by any patient characteristic nor by any side effect. One eye was enucleated, due to untreatable extensive SRF after several vitrectomies and additional bleeding of the tumour.

Indications for the 32 *secondary enucleations* were NVG in 16 patients and tumour recurrence in 10 patients (**Table 3**). Two eyes with 'toxic tumour syndrome' were enucleated and three eyes with severe inflammation (of which one endophthalmitis after cataract surgery). In one of these three patients, this severe inflammation contained necrotic cell debris and no vital tumour cells. The last enucleated eye was in a patient with recurrent retinal detachments and this resulted in a painful blind eye. Larger (T3/T4) tumours were significantly more enucleated (HR: 5.39, 95% 1.25-23.20) compared to smaller tumours. In UM with SRF at baseline (HR: 5.40, 95% CI 1.87-15.47), in eyes with tumour recurrence

(HR: 7.97, 95% CI 3.84-16.51) and NVG (HR: 6.74, 95% CI 3.29-13.79) occurred more enucleations. With multivariable analysis and the variables: TNM classification, SRF at baseline, tumour recurrence and NVG in the model; the only significant factors for enucleation were: NVG ($p < 0.001$) and tumour recurrence ($p < 0.001$; Supplementary **Table 4h**). The median time after treatment for an enucleation was: 41.5 months (IQR: 15.9- 69.3 months). We observed no phthisis, hyphema or scleral melting in eyes with UM after treatment of fSRT.

Visual acuity

At time of diagnosis, the median best corrected visual acuity (BCVA) was 0.15 logMAR (IQR: 0.00-0.51) (**Figure 2**). The visual acuity decreased after treatment: in the first year after diagnosis the median BCVA in logMAR increased from 0.15 to 0.40 (IQR: 0.10-1.00) after three months, 0.45 (IQR: 0.10-1.30) after six months, 0.52 (IQR: 0.15-1.30) after nine months and 0.49 (IQR: 0.15-1.52) after 12 months respectively. The BCVA in logMAR observed at diagnosis was significantly lower and subsequently better vision than one year after fSRT ($p < 0.001$, Wilcoxon signed rank test), than five years after fSRT ($p < 0.001$), than 10 years after fSRT ($p < 0.001$) and, finally, than 15 years after fSRT ($p = 0.004$), respectively. Five years after treatment, 22.0% of patients and 10 years after treatment, 17.6% of patients have visual acuity of ≤ 0.3 logMAR.

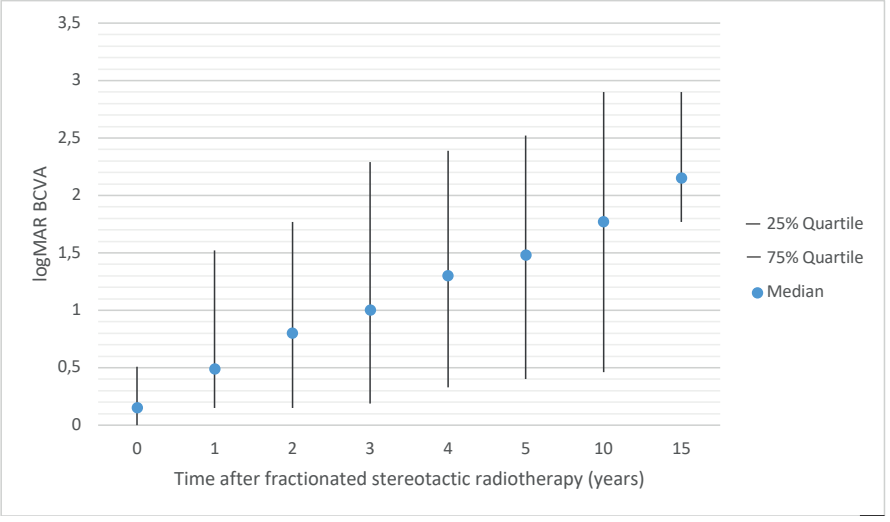

Figure 2. The visual acuity decreases for uveal melanoma patients after fractionated stereotactic radiotherapy treatment. The median Best Corrected Visual Acuity (BCVA) measured in LogMAR after fractionated stereotactic radiotherapy for uveal melanoma increases over time.

Disease-free survival

The cumulative incidence of disease-free survival at 1, 3, 5, 10, 15-years were 96.3%, 81.5%, 77.0%, 69.9% and 61.9% respectively. Univariable analyses showed the development of vitreous haemorrhage (HR: 0.35, 95% CI 0.14-0.87) and retinopathy (HR: 0.53, 95% CI 0.29-0.97) as protective risk factors for death due to UM or for development of metastases. Additionally, SRF at baseline (HR: 3.33, 95% CI 1.62-6.84) and larger tumours (T3/T4) compared to T1 tumours (HR: 6.62, 95% CI 2.04-21.53) were risk factors for worse disease-free survival. Independent risk factors after multivariable analysis (in a model with the variables: SRF at baseline, retinopathy, TNM classification and VH) with death due to UM or metastases as endpoint were: SRF at baseline (p = 0.038) and larger tumours (T3/T4) compared to T1 tumours (p = 0.024; Supplementary **Table 4i**).

DISCUSSION

This study describes local tumour control, radiation induced side effects, visual preservation and disease-free survival after a follow-up of at least five years of UM treated with fSRT. The local tumour control was excellent with cumulative control rates of 99.4%, 92.2%, 89.3% and 89.3% after 1 year, 5, 10 and 15 years, respectively.

Our 5-year local tumour control is higher than the 5-year local progression-free survival of 82 % after fSRT reported by Akbaba *et al.*[12] and than the 2-year local tumour control of 82% after stereotactic radiosurgery/fSRT reported by Yazici *et al.*.[9] On the other hand, our local tumour control was lower than 95.9% after 5 years and 92.6% after 10 years after hypofractionated SRT.[13] In this study tumour recurrence was observed after a median follow-up of 53.2 months. Tumour recurrence was assumed if an increase in tumour volume of more than 25% was observed over two examinations intervals at least 6 months after radiotherapy.[13] Within our study tumour recurrence was observed much earlier at a median follow-up of 19.8 months, and this difference may be due to the used definition for tumour recurrence. In our study we classified tumour recurrence as tumour growth after initial regression after treatment. Overall the 3-year tumour control rate of our study was 92.8% and therefore comparable with other SRT studies with a tumour control rate of 94% at approximately 3 years after SRT[6], and 93.3% after Gamma Knife radiosurgery with a median time of 29.4 months.[14] A higher tumour control rate of 98% has been described after stereotactic external beam radiation with a total study period of 4.5 years.[8] However, in this study, the median follow-up was only 28.3 months and when compared to our study considerably shorter. Although in our study the median time of a recurrence was 19.8 months; the percentages of tumour control are in general difficult to compare as mentioned before, due to differences in tumour characteristics, definitions of tumour recurrence and

follow-up. The overall local tumour control of fSRT (84-98%)[5,8,12,15] is comparable to brachytherapy (82-98%).[16-19]

Radiation induces (late) side effects, depending on the area treated as well as the radiation dose that was used. The five most common side effects in our cohort were cataract followed by retinopathy, maculopathy, VH and neovascular glaucoma. The median time to develop a side effect ranges between 12.1 months for the development of SRF and 29.3 months for the development of maculopathy. This implies frequent follow-up of the patient is necessary, even after two years of treatment.

The lens is sensitive for radiation and cataract is a well-known long-term consequence of radiotherapy. Of the anterior located tumours 85% develop cataract due to the proximity of the lens.[20] In our cohort, we also observed that patients with anteriorly located tumours developed a cataract more frequently. A greater cumulative radiation dose has a direct effect on the development of cataract. However, even after a small dose of 10-18 Gy, cataract has been reported. Our study population has a comparable percentage of cataract (66.5%) compared to other studies that reported 41-75% cataract.[14,21]

The second and third causes of a side effect are radiation retinopathy and maculopathy. Radiation retinopathy gives an altered retinal vascular physiology and shows similarity with diabetic retinopathy. After radiation of the eye high doses of vascular endothelial growth factor (VEGF) are found, which promotes the growth of new blood vessels.[22] In general, the factors that increase the likelihood of developing radiation retinopathy are comorbidities such as diabetes mellitus or hypertension, high radiation dose and proximity of the tumour to the fovea and optic disc.[23,24] UM with posterior margin < 3 mm of the fovea are noted to have early onset and increased severity of retinopathy. We could not confirm that these factors had an effect on the observed retinopathy. This may be due to the fact that the number of cases was too small to reach significance. In addition, Diabetes mellitus and hypertension did not increase the risk of radiation maculopathy and retinopathy in our patient population. An explanation might be that our prevalence of diabetes mellitus was low, only 16 patients. Previously, retinopathy was only treated when patients demonstrated proliferative retinopathy, VH or tractional retinal detachment.[25] Nowadays, treatment starts earlier to retain visual acuity and anatomical structures in the macula, and includes laser photocoagulation, intravitreal anti-VEGF, intravitreal steroids or a combination of those. UM treated after the year 2007 developed more and significantly earlier maculopathy than before 2007. This could be explained by the increasing quality or higher resolution of the spectral-domain (SD) OCT scan and therefore a better detection of this side effect. Radiation maculopathy is on average detectable on OCT at 12 months,

and as early as four months after treatment.[25] After the introduction of anti-VEGF intravitreal injections, maculopathy became treatable and even preventable. In a randomized clinical brachytherapy study in UM patients with radiation maculopathy injections of Aflibercept every six weeks, it appears to limit vision loss and reduced central retinal thickness after one year.[25] And another plaque radiotherapy study with prophylactic anti-VEGF injections of Bevacizumab every four months for two years reported a reduction in maculopathy and better visual acuity compared to a cohort between 2007-2009 without intravitreal injections.[26]

Tumour necrosis, proliferative radiation retinopathy and posterior vitreous detachment have been suggested as a presumed aetiology for VH.[27] Risk factors for development of VH after plaque radiotherapy are the presence of diabetic retinopathy at first visit, shorter tumour distance to the optic disc, greater initial tumour thickness and break in the Bruch membrane.[27] We could not confirm the relation between these risk factors and VH.

Painful eyes with NVG are an indication for secondary enucleation. Of the 16 eyes that had to be enucleated due to NVG, nine developed NVG before the start of intravitreal anti-VEGF injections and seven after diverse treatments. Compared with a study population that started in 1993 that had a 27.3% NVG, our NVG rate is lower.[14] Other studies report 20-42% of NVG.[6,7,28] Larger tumours are associated with neovascular glaucoma and have a higher risk of secondary enucleation.[28,29] In larger tumours, the volume of the irradiated eye is larger, which may increase the risk of side effects. Ischemic changes that end up in neovascularization, especially in larger UM, are a risk for NVG.[30] Transscleral resection of a large UM or with a thickness < 6 mm could be considered to reduce NVG.[31,32] Or endoresection of the tumour in selected patients (tumor diameter > 10 mm and thickness > 5 mm) after proton beam radiotherapy showed less NVG and secondary enucleation.[33] However, in the historical cohort in our study, these treatment options had not yet been available in our institute.

The median BCVA rises from pre-treatment 0.15 logMAR to 0.49 logMAR at 12 months after fSRT. After four years the median visual acuity became worse than 1.3 logMAR in 61 patients. Eleven out of these 61 patients already started with a higher BCVA in logMAR. In total, in 50 patients (42.7%) the BCVA became worse after four years after treatment. This was mainly due to cataract, maculopathy, retinopathy, optic neuropathy, vitreous haemorrhages or neovascular glaucoma. Visual acuity is most effectively preserved in eyes with small tumours outside a radius of 5 mm from the optic disc and fovea.[2]

The cumulative incidence of disease-free survival of 77.0% after 5 years is lower than Yazici *et al.*[9] and Gallie *et al.*[35] report, however comparable with the study of Cohen *et al.*.[34] In our cohort, patients with large tumours and UM with SRF at baseline had a worse disease-free survival. The risk for metastasis and death increased twofold with each increasing tumour category, and the 10-year metastatic rate was 15% for T1, 25% for T2, 49% for T3, and 63% for T4 tumours.[36] Every retrospective study has its flaws, and we had to exclude four patients due to incomplete medical records. On the other hand, our study comprises a period of 15 years after treatment of fSRT and all patients had a follow-up of at least five years. In this period treatment options alter, especially intravitreal injections with anti-VEGF for (the prevention of) ischemic side effects, such as maculopathy, retinopathy and NVG are now standard care. Some patients develop side effects after 10 years of follow-up and studies with a shorter follow-up do not monitor these side effects. This is probably one reason why the incidence of side effects, such as retinopathy, shows wide differences of 2.9% to 41.7% between 12 studies with a follow-up ranging from 6 months to 67 months.[14]

In conclusion, this study presents a long follow-up of the radiation induced side effects of UM treated with fSRT in a tertiary referral centre in the Netherlands. The cumulative 5-year local tumour control was 92.2% and the most common side effects were cataract (67.8%) followed by retinopathy (35.1%), maculopathy (23.8%), vitreous haemorrhage (20.1%), neovascular glaucoma (20.0%) and optic neuropathy (12.4%). By multivariable analysis, risk factors for a worse DFS were larger UM ($p = 0.024$) and tumours with SRF at baseline ($p = 0.038$). The 5-years DFS was 77.0% and visual acuity decreased significantly after start treatment. After 5 years, 22.0% of patients and after 10 years 17.6% of patients had a good vision.

SUPPLEMENTARY TABLES OF MULTIVARIABLE ANALYSIS

Table 4a. Multivariable analysis of the side effect: Tumour recurrence

	p-value	HR	95% CI
Enucleation	<0.001*	10.961	3.299-36.419
SRF at baseline	0.340	2.141	0.449-10.210

HR = hazard ratio, CI = confidence interval, * = significant, SRF = subretinal fluid

Table 4b. Multivariable analysis of the side effect: Neovascular glaucoma

	p-value	HR	95% CI
Enucleation	<0.001*	6.069	3.073-11.988
TNM class	0.309		
TNM class (T2)	0.535	1.490	0.423-5.253
TNM class (T3/T4)	0.197	2.262	0.655-7.811
Age at diagnosis	0.830	0.997	0.971-1.024

HR = hazard ratio, CI = confidence interval, * = significant, TNM class = Tumour Nodes Metastases classification, T2 = UM in thickness classification 2, T3/T4 = UM in thickness classification 3 and 4

Table 4c. Multivariable analysis of the side effect: Cataract

	p-value	HR	95% CI
Margin to fovea	0.047*	1.124	1.002-1.262
TNM class	0.239		
TNM class (T2)	0.351	1.388	0.697-2.766
TNM class (T3/T4)	0.108	1.784	0.881-3.613
Gender	0.092	1.447	0.942-2.224
Age at diagnosis	0.715	0.997	0.979-1.015
Margin to disc	0.241	0.932	0.828-1.049

HR = hazard ratio, CI = confidence interval, * = significant, TNM class = Tumour Nodes Metastases classification, T2 = UM in thickness classification 2, T3/T4 = UM in thickness classification 3 and 4

Table 4d. Multivariable analysis of the side effect: Vitreous haemorrhage

	p-value	HR	95% CI
Age at diagnosis	0.031*	0.970	0.944-0.997
Hypertension	0.066	0.400	0.151-1.063
Gender	0.324	1.400	0.717-2.734

HR = hazard ratio, CI = confidence interval, * = significant

Table 4e. Multivariable analysis of the side effect: Optic neuropathy

	p-value	HR	95% CI
Pigmentation of UM	0.008*	0.295	0.120-0.728
Age at diagnosis	0.149	0.976	0.944-1.009
Margin to disc	0.116	0.849	0.692-1.042

HR = hazard ratio, CI = confidence interval, * = significant

Table 4f. Multivariable analysis of the complication: Radiation maculopathy

	p-value	HR	95% CI
Treatment after 2007	0.003*	2.672	1.392-5.131
Age at diagnosis	0.671	1.005	0.980-1.031
Gender	0.957	0.984	0.543-1.784

HR = hazard ratio, CI = confidence interval, * = significant

Table 4g. Multivariable analysis of the side effect: Radiation retinopathy

	p-value	HR	95% CI
Age at diagnosis	0.007*	0.968	0.946-0.991
Margin to fovea	0.483	1.051	0.914-1.210
TNM class	0.063		
TNM class (T1)	0.021*	2.359	1.141-4.876
TNM class (T2)	0.456	1.261	0.686-2.319
Margin to disc	0.766	0.977	0.841-1.136

HR = hazard ratio, CI = confidence interval, * = significant, TNM class = Tumour Nodes Metastases classification, T1 = UM in thickness classification 1, T2 = UM in thickness classification 2

Table 4h. Multivariable analysis of secondary enucleation

	p-value	HR	95% CI
NVG	<0.001*	4.823	2.231-10.425
Tumour recurrence	<0.001*	6.270	2.839-13.849
SRF at baseline	0.066	2.810	0.935-8.439
TNM class	0.409		
TNM class (T2)	0.421	1.880	0.404-8.750
TNM class (T3/T4)	0.221	2.613	0.562-12.155

HR = hazard ratio, CI = confidence interval, * = significant, NVG = neovascular glaucoma, SR =subretinal fluid, TNM class = Tumour Nodes Metastases classification, T2 = UM in thickness classification 2, T3/T4 = UM in thickness classification 3 and 4

Table 4i. Multivariable analysis of disease-free survival

	p-value	HR	95% CI
SRF at baseline	0.038*	2.199	1.043-4.635
TNM class	0.002*		
TNM class (T2)	0.575	1.440	0.403-5.149
TNM class (T3/T4)	0.024*	4.104	1.209-13.926
Retinopathy	0.142	0.629	0.339-1.167
VH	0.084	0.439	0.173-1.116

HR = hazard ratio, CI = confidence interval, * = significant, SRF = subretinal fluid, VH = vitreous haemorrhage, TNM class = Tumour Nodes Metastases classification, T2 = UM in thickness classification 2, T3/T4 = UM in thickness classification 3 and 4

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CHAPTER 8

LOCAL TUMOUR CONTROL AND RADIATION SIDE EFFECTS FOR FRACTIONATED STEREOTACTIC PHOTON BEAM RADIOTHERAPY COMPARED TO PROTON BEAM RADIOTHERAPY IN UVEAL MELANOMA

Jackelien van Beek*
Wishal Ramdas*
Martina Angi
Caroline van Rij
Nicole Naus
Andrzej Kacperek
Roger Errington
Bertil Damato
Heinrich Heimann
Emine Kiliç
*shared first author

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ABSTRACT

Purpose. To compare the adverse side effects of fractionated stereotactic photon beam radiotherapy (fSRT) with proton beam radiotherapy (PBR) in patients with uveal melanoma (UM).

Methods. A retrospective study investigating 306 UM patients treated with fSRT (n=153) by the Rotterdam Ocular Melanoma Study group (ROMS), The Netherlands, between 1999–2014 or with PBR (n=153) at the Royal Liverpool University Hospital and the Clatterbridge Cancer Centre, Bebington, United Kingdom, between 1993–2014. The tumours treated with fSRT were matched with tumours treated with PBR based on sex, left or right eye, TNM classification, posterior margin ≤ or > 3 mm of the fovea and of the optic disc.

Results. The five-year actuarial rates of tumour recurrence were 4.5% for fSRT and 6.1% for PBR. For fSRT and PBR, the five-year actuarial rates of maculopathy were 14.9% and 12.4%, and for vitreous haemorrhage were 29.4% and 4.7%, respectively. Only vitreous haemorrhage (HR: 0.19, 95% CI: 0.07–0.56) was more common after fSRT compared to PBR. Overall, larger tumours were risk factors for maculopathy and secondary enucleation.

Conclusions. Both treatments have excellent local tumour control. In matched groups, vitreous haemorrhage was the only adverse side effect showing a significant difference between groups.

INTRODUCTION

Fractionated stereotactic photon beam radiotherapy (fSRT) and proton beam radiotherapy (PBR) are both eye-sparing forms of radiotherapy to treat uveal melanoma (UM). These therapeutic modalities provide excellent tumour-control while conserving the eye, usually with useful vision.[1-3] Several studies have concluded that radiotherapy is as effective as enucleation concerning metastatic disease and death.[4,5] Therefore, enucleation is now reserved for eyes with a tumour deemed too large for radiotherapy.[6]

FSRT is suitable for small- and medium-sized melanomas up to approximately 12 mm in thickness and a diameter less than 16 mm. An advantage of fSRT over PBR is that it does not require surgical insertion of fiducial markers for tumour localization and that it is more readily available than PBR. Reported complications of fSRT are neovascular glaucoma, cataract, vitreous haemorrhage, optic neuropathy, maculopathy, retinopathy, and secondary enucleation is required in 3–16% patients.[1,7-9] Local tumour control rates have been reported as high as 96–100% after fSRT.[1,7,8,10]

PBR is available in a growing number of centres in Europe. Some ocular oncologists administer this treatment to all patients, while others reserve it for patients whose tumour is considered unsuitable for brachytherapy. With PBR, radiation is delivered homogenously to the tumour with the dose rapidly falling to zero distal to the tumour.[11] PBR is generally reserved for tumours not exceeding 20 mm in diameter and/or 12 mm in thickness, so as to avoid severe exudative and neovascular complications resulting in a blind and painful eye ('toxic tumour syndrome').[12] The reported local tumour control rates are 96% at 5 years and 94– 95% at 10 years after PBR.[13-15] Secondary enucleation rates are 5–16% due to local recurrence or toxic tumour syndrome.[14,16,17] Ocular morbidity can also occur as a result of collateral damage to lens, optic nerve, macula and development of glaucoma or vitreous haemorrhage.[15,18]

Although, outcomes of fSRT and PBR for UM have been evaluated, fSRT and PBR have not previously been compared with respect to ocular outcomes of matched data in a large study population.[19] As treatment indications, tumour control rates and adverse side effects overlap between fSRT and PBR, we conducted a retrospective study to compare fSRT with PBR for UM with respect to local tumour control and ocular morbidity.

MATERIALS AND METHODS

A retrospective study was conducted in 163 patients with choroidal and/or ciliary body UM treated with fSRT by the Rotterdam Ocular Melanoma Study group (ROMS), The Netherlands, between December 1999 and January 2014, and 912 patients treated with PBR in the Royal Liverpool University Hospital, United Kingdom, between January 1993 and January 2014 in Liverpool. In order to assess the differences in survival and to study the complications between two treatments (fSRT and PBR) for UM, we matched both cohorts of each 153 patients. The local medical ethical committees of both institutes approved the study protocol. Informed consent was obtained prior to treatment and the study was performed according to guidelines of the Declaration of Helsinki.

Patients were diagnosed with UM by ophthalmic examination and underwent full systemic examination. The fSRT patient data were collected in a customised database application based on Filemaker 16 (FileMaker Inc, Santa Clara, California, United States). All clinical data and follow-up data were collected for PBR and fSRT patients. UM were categorised according to the TNM classification, 8th edition.[20] This classification is the same as the 8th edition of the AJCC Classification of posterior uveal melanoma, T category.[21] The following adverse outcomes were documented: local recurrence, neovascular glaucoma, vitreous haemorrhage, optic neuropathy, maculopathy and enucleation. Neovascular glaucoma presented with open or closed angle, depending on the extent of neovascularization. Optic neuropathy was defined as visual loss caused by collateral optic nerve damage and diminished colour vision (tested with Ishihara plates) with or without an afferent pupillary defect. Maculopathy was diagnosed by the presence of haemorrhages, hard exudates, and (non)-cystoid edema, which was identified by ophthalmoscopy, optical coherence tomography or fluorescein angiography when available. We excluded cystoid macula edema developing after cataract extraction. Local recurrence of UM was determined clinically with or without ultrasonography and by sequential fundus photography.

The fSRT and PBR protocols have been described previously.[1,3] The stereotactic radiation dose is given in 5 fractions of 10 Gray (total 50 Gray), at the 80% isodose over five consecutive days and the proton radiation dose is 53 proton Gray in 4, daily fractions.

Statistical Analysis

Matching was based on the following variables: age, sex, TNM classification, tumour distances to the fovea and optic disc. For age we applied a window of 5 years, however the other variables required an exact match. As a consequence,

10 of the 163 patients treated with fSRT could not be matched and were excluded; this resulted in 153 fSRT and 153 PBR patients. Differences in complications (i.e., after treatment) between patients treated with fSRT and PBR were analysed using independent t-tests and Chi-square statistics.

The risk of a complication caused by a tumour characteristic was analysed by applying Cox proportional hazard models in the unmatched complete dataset to calculate hazard ratios (HR) with corresponding 95% confidence intervals (CI). Follow-up duration, used as the time variable, was measured from the date of treatment to the latest visit. The models were adjusted for age, sex, and often for type of treatment (i.e., fSRT or PBR). The risk of a complication caused by treatment (fSRT of PBR) was analysed in a matched dataset (n = 306) by applying "non-conditional" and conditional Cox proportional hazard models. Follow-up duration was used as the time variable.

Cumulative incidence analyses were performed on the matched dataset and the log-rank test was used to assess statistical significance between the curves. Actuarial rates were calculated at 1, 3, 5, and 10 years of follow-up. We used complete case analysis and considered p-value ≤ 0.05 as statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 22.0 for Windows (SPSS inc., Chicago, IL, USA) and R statistical package version 3.6.1 for Mac (http://www.r-project.org).

RESULTS

Our study cohort included, 153 UM patients of whom were treated with fSRT and 153 with PBR (**Table 1**). The two treatment groups were matched, based on the significant differences in tumour characteristics between the fSRT and PBR group.

Table 1. General characteristics of the study population and complications after treatment with fSRT or PBR

	fSRT (n = 153)	PBR (n = 153)	P-value
Age (mean± SD) in years	61.8± 11.1	61.6± 10.6	
Female (n (%))	72 (47.1)	72 (47.1)	
Tumor characteristics			
TNM class (n (%))			
1	34 (22.2)	34 (22.2)	
2	64 (41.8)	64 (41.8)	
3	55 (35.9)	55 (35.9)	
4	0 (0.0)	0 (0.0)	
Margin to the fovea ≤ 3 mm (n (%))	88 (57.5)	88 (57.5)	
Margin to the optic disc ≤ 3 mm (n (%))	77 (50.3)	77 (50.3)	
Complications			
Recurrence (n (%))	9 (5.9)	7 (4.6)	0.798
Neovascular glaucoma (n (%))	27 (17.6)	13 (8.5)	0.027
Vitreous haemorrhage (n (%))	28 (18.3)	5 (3.3)	<0.001
Optic neuropathy (n (%))	11 (7.2)	14 (9.2)	0.677
Maculopathy (n (%))	17 (11.1)	19 (12.4)	0.859

fSRT = fractionated stereotactic photon beam radiotherapy
PBR = proton beam radiotherapy
SD = standard deviation

The median follow-up times of the fSRT and PBR groups were 58.5 months (IQR: 26.1–95.2 months) and 40.0 months (IQR: 19.1–70.0 months) respectively ($p < 0.001$). The 5-year local tumour control rates were 96.1% for fSRT and 96.1% for PBR. At the end of the study, the local tumour control was 94.1% after fSRT with 15 years of follow-up and 95.4% after PBR with 15 years (and 20 years) of follow-up, respectively ($p = 0.798$; **Table 1**). The actuarial rates of tumour recurrence are presented in **Table 2**. The median interval between fSRT and tumour recurrence (n = 9) was 19.8 months (IQR: 14.0–72.7 months). The median interval between PBR and tumour recurrence (n = 7) was 29.4 months (IQR: 15.3–36.7 months). Three tumours treated with fSRT developed a recurrence after more than five years (i.e., after 5.3, 6.8 and 7.0 years). These were T3, T2 and T1 class tumours of

the TNM classification, respectively. One T1 tumour treated with PBR developed a recurrence after more than five years (i.e., after 9.2 years). However, greater tumour size and tumour location (≤ 3 mm to the fovea and ≤ 3 mm to the optic disc) were not associated with a higher incidence of tumour recurrences irrespective of treatment (**Table 3**). Furthermore, local tumour recurrence was not significant associated with a type of treatment (**Table 4**). Secondary enucleation for tumour recurrence was performed in 8 (5.3%) patients after fSRT and 3 (2.0%) after PBR. One fSRT patient with a tumour recurrence received additional fSRT. Four PBR patients with tumour recurrence received additional treatment, such as trans pupillary thermotherapy, iodine plaque radiotherapy, ruthenium plaque radiotherapy or adjunctive PBR. The 5-year enucleation rate is higher in patients treated with fSRT (12.4%) than in patients treated with PBR (5.9%). Multivariate analyses showed in the study population (n = 306) that the incidence of vitreous haemorrhages (VH) was significantly higher after fSRT than PBR (HR: 0.19; 95% CI 0.07–0.56) ($p < 0.0001$) (**Table 4** and **Figure 1**). **Figure 1** shows the cumulative incidence of all complications and complications during follow-up.

Table 2. Actuarial rates (%) of complications at 1, 3, 5, and 10 years of follow-up for fSRT and PBR

	1 year	3 years	5 years	10 years
fSRT				
Recurrence	0.65	3.49	4.52	9.84
Neovascular glaucoma	3.33	15.65	20.22	24.54
Vitreous haemorrhage	9.29	22.21	29.42	29.42
Optic neuropathy	1.32	7.50	8.52	8.52
Maculopathy	2.65	8.85	14.94	14.94
PBR				
Recurrence	0.00	3.62	6.06	11.93
Neovascular glaucoma	1.43	10.24	11.58	14.04
Vitreous haemorrhage	2.25	3.29	4.69	4.69
Optic neuropathy	3.08	11.91	13.15	13.15
Maculopathy	2.93	10.99	12.43	30.37

fSRT = fractionated stereotactic photon beam radiotherapy
PBR = proton beam radiotherapy

Table 3. The risk of a complication caused by a tumor characteristic in the study population (n = 306). Presented as hazard ratio with corresponding 95% CI*

Reference level	TNM-classification 1	Margin to the fovea ≤ 3 mm	Margin to the optic disc ≤ 3 mm
Recurrence	1.69 (0.81-3.51)	1.36 (0.50-3.70)	2.46 (0.77-7.88)
Neovascular glaucoma	1.50 (1.00-2.34)	2.04 (1.07-3.90)#	1.40 (0.72- 2.70)
Vitreous haemorrhage	0.94 (0.62-1.41)	0.93 (0.48-1.78)	0.83 (0.43-1.59)
Optic neuropathy	1.31 (0.77-2.23)	0.72 (0.31-1.68)	0.24 (0.09-0.62)#
Maculopathy	1.99 (1.21-3.26)#	0.96 (0.48-1.91)	1.52 (0.75-3.09)
Enucleation	1.91 (1.14-3.22)#	1.00 (0.50-2.01)	1.20 (0.59-2.44)

CI = confidence interval
* = adjusted for age and gender
# = significant values

Table 4. The risk of a complication caused by treatment (fSRT or PBR; fSRT served as the reference) in the matched study population. Presented as hazard ratio with corresponding 95% CI*

	Matched study population (n = 306)
Recurrence	1.50 (0.42-5.32)
Neovascular glaucoma	0.50 (0.23-1.11)
Vitreous haemorrhage	0.19 (0.07-0.56)#
Optic neuropathy	1.67 (0.61-4.59)
Maculopathy	1.18 (0.53-2.64)
Enucleation	0.47 (0.19-1.15)

fSRT = fractionated stereotactic photon beam radiotherapy
PBR = proton beam radiotherapy
CI = confidence interval
* = adjusted for age, gender, TNM-classification, margin to the fovea, and margin to the optic disc
# = significant values

Regardless of treatment, neovascular glaucoma was 2.0 times significantly more common in tumours that were further than 3 mm of the fovea (**Table 3**). The rate of neovascular glaucoma was higher in fSRT patients (17.6%) than in PBR patients (8.5%) (p = 0.027). The median time to develop neovascular glaucoma was 20.6 months for fSRT patients (IQR: 13.3–33.3 months) and 26.5 months for PBR patients (IQR: 13.2–32.2 months). The actuarial rates of neovascular glaucoma are presented in **Table 2**. After developing neovascular glaucoma, enucleation was required in 4 and 13 patients after PBR and fSRT, respectively.

More fSRT patients (18.3%) developed a VH than PBR patients (3.3%) ($p < 0.001$) (**Table 1**). VH was not associated with tumour characteristics for the total study population (**Table 3**). We found that patients treated with fSRT had significant greater risk of VH compared to PBR (HR 0.19; 95% CI 0.07–0.56) (**Table 4**). The median time to develop a VH was 24.8 months for fSRT patients (IQR: 9.6– 33.4 months) and 11.6 months for PBR patients (IQR: 4.7– 35.0 months). Eleven of the 35 patients treated with fSRT had a VH at baseline. Of those 11 patients, four VH resolved and seven VH remained. After we excluded the seven fSRT patients with a remaining VH from baseline, we found 28 VH that developed after treatment (HR 0.25; 95% CI 0.08–0.75, $p < 0.013$). The actuarial rates of VH are presented in **Table 2**.

Optic neuropathy developed in 7.2% and 9.2% of patients treated with fSRT and PBR, respectively ($p = 0.677$) (**Table 1**). Tumours extending within 3 mm of the optic disc were significantly associated with optic neuropathy (HR: 0.24, 95% CI 0.09–0.62) (**Table 3**). There was no difference in the rate of optic neuropathy between fSRT and PBR (**Table 4**). The median time to optic neuropathy was 17.6 months in fSRT patients (IQR: 12.3–26.4 months) and 18.8 months in PBR patients (IQR: 11.7–28.3 months). The actuarial rates of optic neuropathy are presented in **Table 2**.

Maculopathy occurred in 11.1% and 12.4% of patients after fSRT and PBR, respectively (**Table 1**). T2 and T3 tumours were 2.0 times more likely to develop maculopathy compared to T1 tumours (**Table 3**). There was no difference in the incidence of maculopathy (**Table 4**). The median time to maculopathy was 24.3 months in fSRT patients (IQR: 10.7–49.0 months) and 19.4 months in PBR patients (IQR: 14.2–61.0 months). The actuarial rates of maculopathy after fSRT and PBR are presented in **Table 2**.

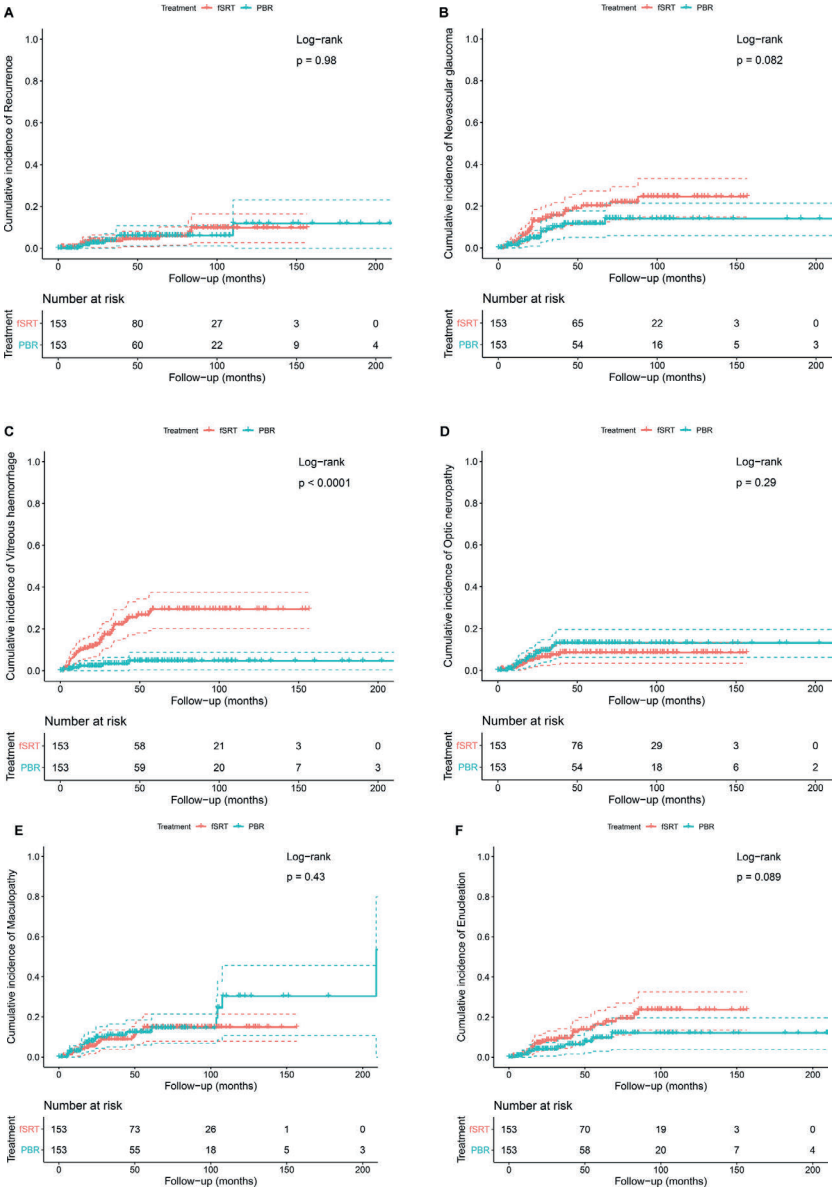

Figure 1. Cumulative incidence analyses with log-rank test on treatment (i.e., fractionated stereotactic photon beam radiotherapy (fSRT) and proton beam radiotherapy (PBR)) for each complication (i.e., recurrence (**A**), neovascular glaucoma (**B**), vitreous haemorrhage (**C**), optic neuropathy (**D**), maculopathy (**E**), and enucleation (**F**)). Censored patients are denoted by vertical tick marks. The dashed lines around the curve represents the confidence intervals for the point estimates of the cumulative incidence curve.

DISCUSSION

To our knowledge, this is the first study to compare outcome of fSRT with PBR as a treatment for UM. We found that high 5-year local tumour control rates were achieved by both methods of radiotherapy treatment (i.e., after 96.1% in PBR patients and 96.1% in fSRT patients). In a matched study population (n = 306), the most common complications were maculopathy (12.4%) after PBR and vitreous haemorrhage (18.3%) after fSRT ($p < 0.001$) (**Table 1** and **4**). Maculopathy and enucleation were significantly and neovascular glaucoma was nearly significant associated with large tumour size. As expected, with tumour proximity to optic disc more optic neuropathy was observed. Neovascular glaucoma was associated with tumours located further than 3 mm of the fovea.

A weakness of the study is that fSRT and PBR were performed in different centres, which may not have measured baseline features and outcomes in the same way. It would have been ideal if both centres had randomised patients, however, neither centre had access to both forms of radiotherapy. As in other retrospective studies, our study may have also suffered from bias caused by missing data and loss of patients from follow-up. In order to compare the different complications of both treatments we performed analyses on matched data to have equal groups regarding: sex, age and tumour characteristics (TNM-classification, tumour distances to fovea and optic disc).

An excellent 5-year tumour control rate was achieved with either treatment and was comparable to previous studies.[15,19,22,23] When we analysed the matched population only VH was significant more common after fSRT than after PBR (**Table 4**). Of note, 11 of the 35 patients treated with fSRT already had VH at baseline; however, this had no influence on the development of new VH after treatment. PBR patients had no VH at baseline, as good tumour visualization was needed to perform clip surgery prior to PBR. We are unable to explain why VH was more common after fSRT than after PBR. This difference may have occurred by chance. In any case, this was not a serious complication as it could easily be treated with vitrectomy. Tumour necrosis, proliferative radiation retinopathy and posterior vitreous detachment have been suggested as a presumed aetiology for VH.[24] In our study we did not take into account the regression rate of the tumour, which might reflect the amount of tumour necrosis and might explain part of the VH. Proliferative radiation retinopathy is not specifically observed within this group of patients. Another study after plaque radiotherapy found underlying diabetic retinopathy, closer tumour proximity to the disc, greater tumour thickness, and break in the Bruch membrane as predictive factors for a VH.[24] In our study we also did not record break of Bruch membrane routinely and did not have complete data on diabetes mellitus status; however, we did not find an effect of tumours closer to optic nerve or tumour thickness as risk factors for vitreous haemorrhages (**Table 3**).

When considering complications of both treatments, we observed that the largest tumour diameter is an important risk factor for adverse outcome. In our population larger tumours required more often an enucleation. This is in contrast to Yazici *et al.*[9] who found no differences in enucleation rates between eyes with large and small/medium tumours (p = 0.2) after stereotactic radiosurgery and fSRT.In another study large T3 and T4 tumours treated with PBR, 19.5% of the tumours were enucleated, which was higher than in our cohort.[25]

We recorded neovascular glaucoma in 8.5% of the PBR-treated patients and 17.6 % in fSRT patients. Most proton beam centres have reported higher percentages of neovascular glaucoma ranging from 12.7% to 47% of the patients.[26,27] FSRT centres report for 24.5–42% neovascular glaucoma.[7,22] A point of attention is that almost 60% of the current study population was in the era before anti-vascular endothelial growth factor (VEGF) intravitreal injections, which could explain the high percentage of neovascular glaucoma in both groups. And with the current treatment options with anti-VEGF we may expect less neovascular glaucoma, however, this has to be evaluated in a prospective study.[28]

A tumour within 3 mm of the optic nerve would result in a high dose of radiation of the optic nerve and consequently would lead to a decrease in visual acuity. Juxtapapillary UM are a risk factor for developing optic neuropathy (**Table 3**). And for those tumours, percentages of optic neuropathy as high as 68% were observed.[29] In tumours treated with fSRT optic neuropathy occurs in 61.5% of patients.[22] In PBR treated eyes comparable and higher percentages (14–68%) of optic neuropathy were found.[19,30] This is in contrast with a study where more than half of their patients had juxtapapillary T3 and T4 UM and only 8.3% developed optic neuropathy.[25] In the end, however, there is no standardized definition of optic neuropathy resulting in different definitions used by different studies.

Maculopathy is another vision threating complication after radiation. In our population, 57.5% of the UM were closer than 3 mm to the fovea. Despite that, only 11.1% of the fSRT patients and 12.4% of the PBR patients developed a maculopathy and this was not related to the tumour distance to the fovea in the current study. Interestingly, as also observed in other studies, maculopathy occurred more often with an increase in the size of the tumour, history of diabetes mellitus and presence of preoperative subretinal fluid.[30,31] Guyer *et al.*[32] observed in 89% of the paramacular tumours maculopathy after PBR. After radiation, high doses of VEGF are found in the eye.[33] The treatment of anti-VEGF intravitreal injections seems to limit visual loss associated with radiation maculopathy, although this was analysed after a different form of radiation treatment with plaque therapy.[28,34] Shields *et al.*[28] observed a decrease in radiation maculopathy with preservation of visual acuity after prophylactic Bevacizumab every four months.

An enucleation was performed more often in eyes with a larger tumour. This might be explained by the fact that patients with peripheral tumours often present rather late and may have consequently a larger tumour.[35] The 5-year overall enucleation rate is higher in patients treated with fSRT (12.4%) than in patients treated with PBR (5.9%). However, there is no significant difference between treatments in the risk of enucleating the eye. When comparing different studies, it is important to keep in mind that the indication of a treatment can differ, as fSRT cannot be performed for small tumours whereas PBR can. In other centers where fSRT is performed, the percentages of enucleation were 13.2–17%.[22,36] The same counts for UM treated with PBR, where 7.7% of the eyes were enucleated after 5 years.[15] As the local control in UM patients is high regardless of treatment, the emphasis must lie on limiting the ocular morbidity for patients' quality of life.[37] Moreover, knowledge on the occurrence of complications can help caregivers to apply personalized treatment.

In summary, it can be stated that both treatment options are comparable in their outcome, although fSRT patients developed more vitreous haemorrhages. This is a complication that can be managed very well surgically. As observed in other studies juxtapapillary location has a higher risk of developing optic neuropathy, irrespective of the type of radiation. Overall, in our population, the risk factor for maculopathy and enucleation was the increase in tumour size. A tumour located more than 3 mm from the fovea is more prone to develop neovascular glaucoma.

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CHAPTER 9

QUALITY OF LIFE: FRACTIONATED STEREOTACTIC RADIOTHERAPY VERSUS ENUCLEATION TREATMENT IN UVEAL MELANOMA PATIENTS

Jackelien van Beek
Gabriëlle Buitendijk
Reinier Timman
Karin Muller
Gregorius Luyten
Dion Paridaens
Nicole Naus
Emine Kiliç

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ABSTRACT

Purpose. To report the quality of life and visual functioning in uveal melanoma patients treated with enucleation or fractionated stereotactic radiation therapy (fSRT).

Methods. Uveal melanoma (UM) patients treated with fSRT (n = 65) or enucleation (n = 48) participated in this prospective study. Questionnaires to measure anxiety (State-Trait Anxiety Inventory), subjective distress (Impact of Event Scale) and quality of life (EORTC QLQ-C30 and National Eye Institute Visual Function Questionnaire (VFQ-25)) were obtained before treatment and 2, 6, 12, 24, 36 and 48 months after treatment.

Results. Less peripheral vision was observed until 3 years ($p = 0.026$) posttreatment in enucleated patients compared to irradiated patients. From 2 months until 3 years posttreatment irradiated patients increase in role functioning-score ($p = 0.005$), while enucleated patients decrease in score ($p = 0.012$). Regardless of their treatment, for all patients we measured a reduction in physical functioning ($p = 0.035$), insomnia ($p < 0.001$) and in state anxiety from pretreatment until 2 years posttreatment ($p < 0.001$). An increase in pain overall ($p = 0.023$) and in emotional functioning is observed 1 year posttreatment ($p < 0.001$). At baseline, patients with metastases (independent of their treatment) have more subjective distress ($p = 0.037$) than patients without metastases. The mean 'global health score' overall, without effect of time, was 76.4 (SD: 13.6).

Conclusions. Enucleated patients had more difficulty working or performing household tasks 2 months posttreatment compared to irradiated patients. Enucleated patients had diminished peripheral vision until 3 years compared to irradiated patients. Overall quality of life is not significantly different between both treatment groups.

INTRODUCTION

Quality of life (QoL) is highly subjective and a dynamic process, subject to changes in life and life events. Yearly, 6-7 people per million are diagnosed with uveal melanoma (UM).[1] The survival is generally not influenced by the treatment strategy chosen for the primary tumour.[2] In the early days, enucleation was the standard treatment for UM. Nowadays, several eye sparing treatment modalities are available with the main purpose of sparing visual function. The decision to have the eye enucleated is inevitable in patients with very large tumours and can be psychologically traumatic. Enucleation and irradiation treatment have different psychological effects on patients, which reflects quality of life.[3,4] However, studies on the exact effect are inconclusive. Questionnaires have been developed to measure patients' wellbeing and could assist, to customize treatment for each patients' need.

Cancer diagnosis led in general to quality of life impairment compared to healthy subjects and patients with other non-oncological ophthalmological conditions.[5] Treatment of UM seemed to reduce quality of life at least in the first 3 years after diagnosis.[3,6] However, impaired as well as normal quality of life has been found within a 5-year follow-up.[7,8] Nevertheless, 5 years after diagnosis and treatment for UM, quality of life is higher compared to other cancer types.[9] In addition, patients have reported about fear of local recurrence equally after enucleation and conservative treatment.[10] Twenty patients in one study revealed that the worst psychological moment (mild to severe state of depression) after enucleation occurred 3 months after surgery. They reported more difficulties in adaptation and more anxiety than after 1 year. One year after enucleation, they appeared to have a more balanced quality of life.[11] Enucleated patients reported problems with appearance and judging distances one year after treatment.[3] In addition, 72-85% of the patients report a reduced quality of life and 60-74% experience reduced emotional functioning irrespective of their treatment with Ruthenium plaque or enucleation.[3] While comparison of the mean levels of quality of life before and after treatment with LINAC, Ruthenium-106 and Gamma Knife showed only 5% decline.[6] Patients treated with brachytherapy are significantly better in driving and have better peripheral vision than enucleated patients for up to 2 years following treatment.[4]

Non-invasive fractionated stereotactic radiation therapy (fSRT) was introduced in our clinic in 1999, as a locoregional treatment for medium and large-sized UM with promising results for tumour control without serious side-effects.[12] As enucleation and fSRT offer comparable survival rates, the assessment of patients' physical, mental and overall patients' quality of life is of great importance in treatment decision.[13,14] There are only few studies that have compared the quality of life of uveal melanoma patients treated with fSRT or enucleation.[6] Therefore, a prospective study was initiated in our clinic to compare the quality of life in patients who underwent an enucleation versus patients treated with fSRT.

PATIENTS AND METHODS

Design

From February 1st, 2002, all UM patients who visited the tertiary ocularoncology clinic were offered to participate in this quality of life study. All questionnaires were sent to the patients and filled in at home or in the clinic during their follow-up visit. The baseline (pretreatment) measurement took place at the second visit. After starting either treatment (enucleation or fSRT), the questionnaires have been taken after two, six and 12 months, followed by a questionnaire once a year for 4 years after treatment. The first patient was treated on March 8th, 2002 and the latest patient was treated on December 2nd, 2009. This study was approved by the Medical Ethics Committee of the Erasmus MC and was conducted in accordance with the tenants of Declaration of Helsinki. Written informed consent was obtained.

Patients

For fSRT treatment (5 x 10 Gy) a tumour ought to have a thickness smaller than 12 mm and a diameter smaller than 16 mm. Patients with larger tumours underwent enucleation and some patients preferred enucleation after comprehensive counselling about both treatment modalities given by an ophthalmologist and a radiation oncologist. Because of medical-ethical reasons, randomization was not possible. Patients who were initially treated with fSRT and secondarily enucleated were censored at the moment of enucleation. Local recurrence was defined as persisting increase in the tumour on B-scan ultrasonography (US) or enlargement of the tumour on fundoscopy. Disease-free survival (DFS) was defined as the moment of diagnosis until development of metastases or patients' death. Cases in which the cause of death was unknown or not related to their UM were treated as censored. Survival date was obtained until December 1st 2013. Tumour characteristics, the largest tumour diameter (LTD) and the tumour thickness, were measured, using US. Visual acuity of the affected and contralateral eye was measured in fSRT patients and was converted into LogMar.

Questionnaires

The quality of life (QoL) questionnaires included three generic questionnaires, State-Trait Anxiety Inventory (STAI), the Impact of Event Scale (IES), the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30, and one disease specific standardized questionnaire, the 25-item National Eye Institute Visual Function Questionnaire (NEI VFQ-25).

State-trait anxiety inventory (STAI)

The Dutch version of the STAI-version DY is a self-report questionnaire and measures two subscales, state and trait anxiety.[15] The state anxiety scale evaluates the current state of anxiety. The trait anxiety evaluates anxiety level as a personal characteristic. It consists of two series of 20 items each on a 4- point scale, designed to assess various aspects (transient or situational and stable or dispositional) of trait anxiety. Scores range for each subset from 20 to 80, where higher scores correlate with greater anxiety. A cut-off point of 39-40 has been suggested to detect clinically significant symptoms for the state anxiety scale.[16] However, higher cut-off points have been suggested as well.

Impact of event scale (IES)

The Dutch version of the IES was used to measure subjective distress after a specific stressor (in this case being treated for UM).[17-19] It is a two-factor 'intrusion–avoidance' model consisting of a 15-item self-report questionnaire on a 4-point scale. It measures both intrusion (7 items), characterized by unbidden thoughts and images of the event, troubled dreams, strong pangs or waves of feelings and repetitive behaviour, and avoidance (8 items), characterized by denial of meaning and consequences of the event. The weighted sum of the responses is the scale score, ranging from 0 to 75. Cutoff point of low (0-8), medium (9-19) and high (≥20) levels of distress has been suggested.[20]

EORTC QLQ-C30 version 3.0

The EORTC QLQ-C30 is designed for cancer patients and measures the quality of life on multi-item scales, including five functional scales (physical functioning, role functioning, emotional functioning, cognitive functioning and social functioning), a global health/QoL-scale and symptom scales/items about additional symptoms commonly reported by cancer patients (fatigue, nausea/vomiting, pain, dyspnoea, insomnia, appetite loss, constipation, diarrhoea and financial difficulties).[21] All scores range from 0 to 100. The higher scores representing a better quality of life. This is in contrast to the symptom scales. In the symptom scales, higher scores indicate more severe symptoms. Our patients received a Dutch translation of the EORTC QLQ-C30.[22] An example of the role functioning question was for the lowest possible score (0): 'In the past week I was completely unable to work at a job or do household jobs' and was for the highest possible score (100): 'In the past week I was not limited at all doing either work or household jobs'.

National eye institute visual function questionnaire (NEI VFQ-25)

The NEI VFQ-25 is used to measure the influence of the different treatment modalities for UM on patients' functioning and wellbeing.[23,24] It consists of 12 subscales from 39 items with 1-6 items per subscale: 2 items in general health, general vision and ocular pain, 6 items in near activities and distance activities, 3 items in social functioning and driving, 5 items in mental health, 4 items in role difficulties and dependency and 1 item in colour vision and peripheral vision. Each item is converted into a 0-100 scale and a higher score represents better functioning.

Statistical methods

Multilevel hierarchical linear regression analyses were applied for longitudinal analyses of the data. This method can efficiently handle missing data and data with unbalanced time-points. There were two levels in the models. The patients constitute the upper level; their repeated measures the lower level. First, a model was postulated for each outcome variable with linear and logarithm of time, treatment (enucleation or fSRT), the occurrence of metastasis and interactions with time as fixed effects. The deviance statistic using restricted maximum likelihood was applied to determine the covariance structure.[25,26] Effect sizes were calculated from dividing differences between time-point estimations and baseline by the estimated baseline standard deviation. For the interpretation of the effects sizes the definition of Cohen was used: an effect size of 0.20 is considered a small effect, 0.50 medium and 0.80 a large effect.[27] A Cox regression analysis was performed to analyse the difference in DFS between fSRT and enucleation. To correct for the confounding effect of the largest tumour diameter, tumour thickness and treatment on DFS, all factors were entered as a covariate. Statistical analyses were performed with IBM-SPSS release 21 (SPSS Inc, Chicago, Ill).

RESULTS

Patient and tumour characteristics

In total, 116 patients participated in this prospective study. Three patients withdrew after receiving the first questionnaire. The study included 113 patients (60 men and 53 women). Baseline characteristics are shown in **Table 1**. There was no difference in gender between the two groups (p = 0.056, **Table 1**). The overall mean age was 65 years at time of treatment (range 33-89 years). Patients were treated with either fSRT (58%) or enucleation (42%). Thirteen patients treated with fSRT underwent a secondary enucleation due to tumour progression (n = 4) or complications (painful neovascular glaucoma (n = 8), endophthalmitis after cataract surgery (n = 1)) after a median period of 29.2 months (range 9.3-63.4 months). Three patients were

lost to follow-up. One patient stopped after questionnaire 2 due to migration and one patient stopped after questionnaire 5 for unknown reason. The last patient finished the questionnaires, however was lost to follow-up 8 years posttreatment. Questionnaires were included until the last date of follow-up. **Figure 1** illustrates the number of completed questionnaires until 4 years posttreatment. A significant larger mean LTD was observed in tumours treated with enucleation compared to fSRT-treated tumours, respectively 13.4 mm versus 11.9 mm ($p = 0.017$, independent sample t-test). The mean tumour thickness was equal in both groups ($p = 0.061$, independent sample t-test). Patients treated with enucleation metastasized significantly more often than patients treated with fSRT (56% versus 26%, $p = 0.006$, Chi-square test). The disease-free survival in enucleated patients ranged from 5.2-124.6 months and in fSRT patients from 3.2-133.1 months. Univariate analysis showed a significantly shorter mean survival (69.5 months) in patients who underwent an enucleation (95% CI 56.7-83.2, X^2: 12.49, $p < 0.001$, log rank test). However, when corrected for largest tumour diameter and tumour thickness there was no significant difference in survival between both treatment options (Hazard ratio (HR) = 1.78, $p = 0.099$, Cox proportional hazard model). In multivariate analysis, only largest tumour diameter was related with survival (HR = 1.32, $p < 0.001$, Cox proportional hazard model).

Table 1. Baseline characteristics of irradiated and enucleated patients

Patient characteristics	fSRT n = 65	Enucleation n = 48	P-value
Gender, *n* (%)			0.056*
Men	40 (62%)	20 (42%)	
Women	25 (38%)	28 (58%)	
Age in years, mean (SD)	67.0 (±12.1)	63.3 (±12.6)	0.119†
Mean largest tumour diameter, mm (SD)	11.9 (±2.7)	13.4 (±3.9)	**0.017**†
Mean tumour thickness, mm (SD)	6.0 (±2.5)	7.5 (±4.3)	0.061†
TNM classification			
T1 (*n* = 29)	17	12	
T2 (*n* = 35)	24	11	
T3 (*n* = 40)	23	17	
T4 (*n* = 9)	1	8	
Disease free survival (DFS) in months, mean (SD)	77.8 (±36.2)	53.8 (±34.4)	**<0.001**‡
Alive, *n* (%)	35 (54%)	18 (38%)	**0.006***
Metastases, *n* (%)	17 (26%)	27 (56%)	**0.006***
Death other cause, *n* (%)	9 (14%)	3 (6%)	
Lost to follow-up, *n* (%)	4 (6%)	0 (0%)	
Visual acuity, LogMar (SD)			
affected eye	0.526 (0.75)		
contralateral eye	0.043 (0.31)		

SD = standard deviation

* Chi square or Fisher's exact test

† Independent sample t-test

‡ Log rank test

P-values in bold are significant

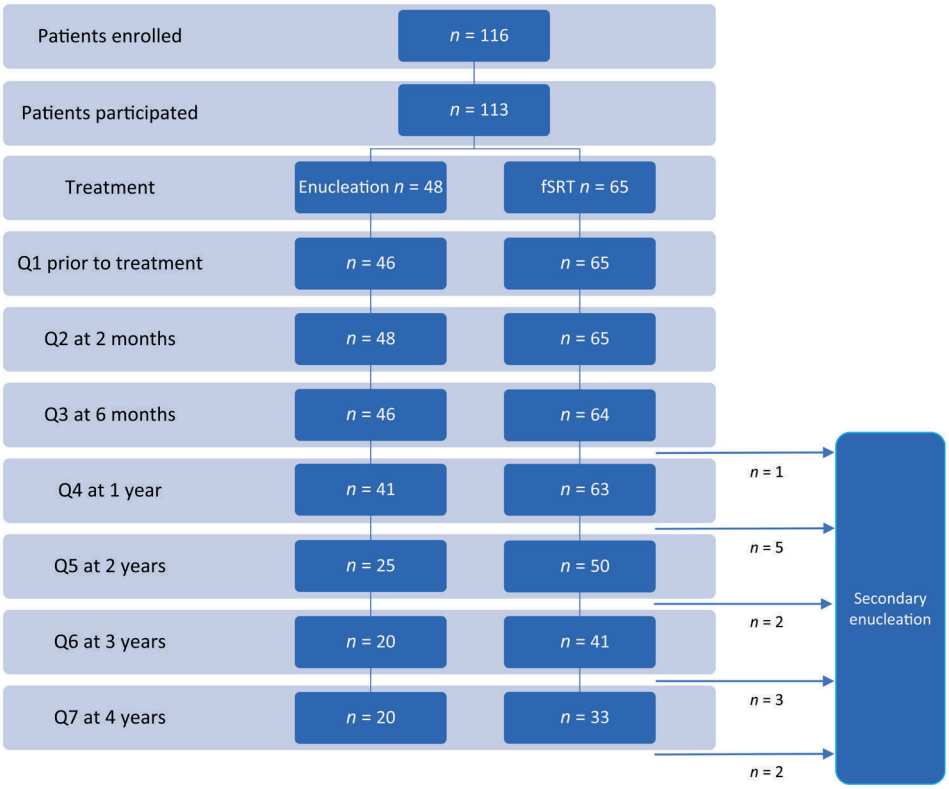

Figure 1. Patients enrolled in this study and number of responses (n = 113) of questionnaires over time. Arrows: the number of patients who underwent a secondary enucleation. fSRT = fractionated stereotactic radiotherapy; Q1-Q7 = moment 1-7 at which the questionnaires were taken after treatment (Enucleation or fSRT)

Anxiety level

STAI

A reduction in state anxiety (the current state of anxiety) was measured from pretreatment until 2 years after treatment in both treatment groups (pretreatment: 44.4, 95% CI 42.3-46.4 and at 2 years 37.5, 95% CI 35.5-39.5, $p < 0.001$). Between pretreatment and until 4 years after treatment a medium effect (Cohen's d: 0.52) was noted. At baseline, fSRT patients scored (37.3) slightly higher in trait anxiety (anxiety level as a personal characteristic) than enucleated patients (35.7). After 2 years, enucleated patients experienced more trait anxiety than patients treated with fSRT, although this effect was not significant ($p = 0.088$). There was no significant difference observed between the two treatment groups for trait

and state anxiety. When we compared the scores of patients with and without metastases, despite their treatment, patients with metastases were significantly more anxious (state anxiety only) after 3 years (p = 0.026, Students' t-test) than patients without metastases (**Table 2**).

Table 2. Significant differences in enucleation and metastases*

	Baseline		12 months		24 months		36 months		48 months	
	d	***p***	***d***	***p***	***d***	***p***	***d***	***p***	***d***	***p***
Enucleation										
Peripheral vision	-0.36	<0.001	-0.53	<0.001	-0.49	<0.001	-0.36	0.026	-0.21	0.460
Metastases										
State anxiety	0.30	0.136	0.21	0.262	0.38	0.064	0.59	0.026	0.81	0.022
Emotional functioning	-0.13	0.498	-0.15	0.415	-0.40	0.062	-0.68	0.022	-0.98	0.018
Nausea	-0.12	0.536	-0.02	0.882	0.23	0.209	0.50	0.054	0.78	0.037
Insomnia	0.28	0.156	0.34	0.058	0.51	0.009	0.70	0.004	0.89	0.006
Appetite loss	-0.02	0.920	0.12	0.483	0.36	0.051	0.63	0.014	0.89	0.012

*Compared to fSRT and respectively no metastases
d = Effect size Cohen's d, p = p-value, Student's t-test

Subjective distress

IES

The 'Intrusion' score as well as the 'Avoidance' score showed no difference for the enucleated and fSRT patients. However, a significant reduction ($p < 0.001$) for 'Intrusion' and 'Avoidance' scores overall, was noted from pretreatment until 4 years after treatment in all patients. After patients were diagnosed, a medium level of distress was measured, which reduced to a low level 6 months after treatment. At baseline, patients with metastases scored only significantly higher in 'Intrusion' (16.5, 95% CI 11.5-21.5, versus 5.28, 95% CI 0.3-10.3, p = 0.037, Students' t-test) than patients without metastases. After this point, we found no significant difference between these two groups.

EORTC QLQ-C30

The scores on the EORTC questionnaire are given in **Table 3**.

Functional scale

The physical functioning subscale showed no difference between the enucleation and fSRT patients before treatment and during follow-up. However, a decrease in physical functioning was significant ($p = 0.035$) over time in both treatment groups. At baseline, no significant difference is measured in the subscale role functioning ($p = 0.183$) between the enucleated and fSRT patients. **Figure 2** shows that after 2 months irradiated patients improve significantly in role functioning-score ($p = 0.005$). On the contrary, enucleated patients showed a significant decrease in role functioning ($p = 0.012$). However, after 4 years no significant difference between fSRT and enucleated patients was measured ($p = 0.063$) in scores. In both treatment groups, emotional functioning subscale improves ($p < 0.001$) from 1 year posttreatment. This effect is the largest after 2 years; Cohen's d is 0.67 after two and 0.53 after 4 years, respectively (medium effect). After 3 years, patients with metastases, regardless of their treatment, scored significantly lower (score 69.9, 95% CI 64.9-74.9) in emotional functioning compared to patients without metastases (score 83.4; 95% CI 78.4-88.4, $p = 0.022$, Student's t-test) (**Table 2**). In the subscales cognitive and social functioning, we found no significant differences between the treatment options and between patients with and without metastases.

Symptom scales and single items

Nausea/vomiting and pain subscales revealed no significant differences between both treatment groups. However, patients with metastases had a significant higher score (10.8, 95% CI 8.9-13.5, versus 2.5, 95% CI -0.2 to 5.2) in nausea after 4 years regardless of their treatment modality ($p = 0.037$, Student's t-test) (**Table 2**). A significant increase in pain score was noted in patients after enucleation and fSRT (pretreatment: 14.3, 95% CI 10.2-18.4, versus 4 years posttreatment: 19.2, 95% CI 15.1-23.3, $p = 0.023$). However, this effect is small (Cohen's d factor: 0.19 after 4 years).

The insomnia and appetite loss subscales showed no difference between the enucleation and fSRT patients. A significant decrease in insomnia was measured over time ($p < 0.001$) for both treatment groups. In contrast, for patients with metastases (independent of which treatment) insomnia increased significantly ($p = 0.009$, Student's t-test) after two years (**Table 2**). The score for appetite loss increased ($p = 0.014$, Student's t-test) after 3 years in this group as well (**Table 2**). Patients with metastases developed more insomnia and experienced more appetite loss with time than patients without metastases. The subscales: fatigue, dyspnoea, constipation, diarrhoea and financial difficulties revealed no differences between both treatment groups. However, patients with metastases developed less financial difficulties and more diarrhoea after 3 years ($p = 0.014$).

In the Global health subscale, we found no difference in quality of life between the two treatment groups. The overall global health score, with no effect of time, was 76.4 (SD 13.6) on a scale of 0-100. No significant difference was observed between the patient groups with and without metastases, however the score after 4 years was lower for patients with metastases (71) versus without metastases (78).

Visual functioning

Visual Function Questionnaire

At baseline enucleated patients scored better on the General health subscale, while 2 years after treatment irradiated patients scored better. The difference between these groups was not significant ($p = 0.09$). The score of fSRT patients declines significantly compared to baseline until 2 years after treatment ($p = 0.007$), and the score of enucleated patients showed a significant ($p < 0.001$) decline after 4 years compared to baseline. In addition, patients with or without metastases showed no significant difference.

A significant difference was noted in the peripheral vision domain from baseline (fSRT: 82.6 versus enucleation: 66.9) until 3 years of follow-up after treatment between the enucleated (58.9) and fSRT (69.3) patients, ($p < 0.001$) until 2 years after treatment, and 3 year after treatment ($p = 0.026$, Student's t-test) (**Table 2**, **Figure 3**). There was no significant difference between metastasized and non-metastasized patients. All the other domains showed no significant difference between enucleated and fSRT patients with UM and between patients with and without metastases (despite their treatment).

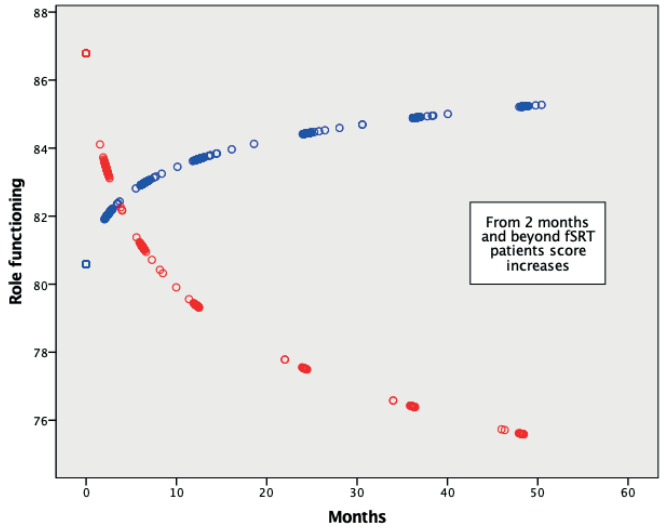

Figure 2. Subscale role functioning measured in EORTC QLQ-C30. Patients treated with fSRT (blue dots) increase significant (p = 0.005) after 2 months in role functioning compared with enucleated patients (red dots).

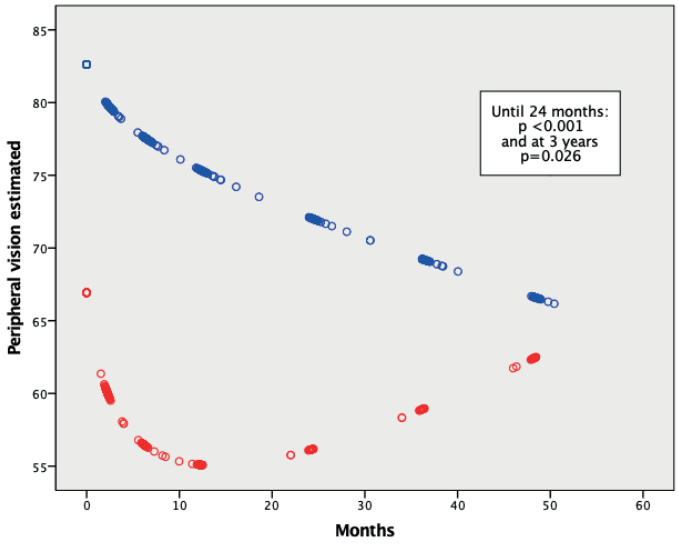

Figure 3. Peripheral vision score of the VFQ-25 questionnaire. A significant difference is noted in peripheral vision from baseline until 3 years after treatment between the enucleated (red dots) and fSRT (blue dots) patients in the first 2 years. Thereafter, there was no significant difference between the two treatment options.

Table 3. The raw data of the EORTC questionnaire

EORTC	Pre-treatment (fSRT) (n = 65)	Pre-treatment (enucleation) (n = 46)	fSRT (n = 65)	Enucleation (n = 48)	fSRT (n = 64)	Enucleation (n = 46)
	Baseline (form 1)		2 months (form 2)		6 months (form 3)	
Global health status/ QoL Mean (SD)	76.4 (13.6)	76.1 (13.5) **(n = 45)**	75.8 (13.3) **(n = 64)**	78.1 (13.9)	76.7 (13.9)	76.3 (14.1) **(n = 45)**
Functional scales						
Physical functioning Mean (SD)	85.9 (19.6)	85.7 (23.4)	84.2 (19.6)	80.7 (22.6)	83.5 (21.9)	82.7 (23.0)
Role functioning Mean (SD)	80.0 (29.1)	87.0 (22.2)	82.3 (27.1)	81.9 (26.4) **(n = 47)**	84. 4 (28.0)	88.0 (24.0)
Emotional functioning Mean (SD)	69.0 (22.7)	64.2 (20.4) **(n = 45)**	81.0 (19.0)	83.5 (14.0)	79.9 (21.1)	80.7 (17.3) **(n = 45)**
Cognitive functioning Mean (SD)	80.8 (21.5)	81.9 (17.7) **(n = 45)**	83.6 (21.8)	85.1 (18.3)	84.1 (18.4)	87.4 (17.1) **(n = 45)**
Social functioning Mean (SD)	92.6 (12.5)	89.4 (18.0) **(n = 44)**	91.0 (14.5)	91.7 (16.1)	90.9 (17.6)	90.0 (16.8) **(n = 45)**
Symptom scales/items						
Fatigue Mean (SD)	23.3 (23.7)	21.1 (20.4)	26.1 (24.7)	21.6 (18.6)	24.0 (22.0)	23.3 (19.2)
Nausea/ Vomiting Mean (SD)	5.1 (11.0)	4.0 (9.4)	3.1 (9.7)	1.7 (6.2)	2.9 (8.2)	3.3 (8.3)
Pain Mean (SD)	14.1 (22.3)	10.9 (18.3)	17.2 (23.0)	12.9 (20.1)	18.5 (24.2)	16.7 (24.9)
Dyspnoea Mean (SD)	8.2 (16.7)	4.4 (13.4)	12.3 (22.5)	5.6 (12.6)	10.4 (18.7)	9.4 (18.1)
Insomnia Mean (SD)	17.2 (32.8)	28.2 (27.5) **(n = 45)**	26.2 (33.6)	27.0 (28.4) **(n = 47)**	22.4 (27.3)	23.2 (26.2)
Appetite loss Mean (SD)	8.7 (21.5)	10.9 (18.7)	3.1 (11.4)	7.7 (17.2)	5.7 (15.2)	5.8 (16.2)
Constipation Mean (SD)	4.6 (14.3)	5.2 (14.1) **(n = 45)**	5.2 (14.8) **(n = 64)**	8.3 (20.1)	5.2 (18.0)	6.7 (15.2) **(n = 45)**
Diarrhoea Mean (SD)	6.2 (14.3)	5.3 (14.3) **(n = 44)**	6.2 (16.6)	2.8 (9.3)	3.1 (9.8)	6.7 (16.8) **(n = 45)**
Financial difficulties Mean (SD)	4.1 (13.8)	0.8 (5.0) **(n = 45)**	2.6 (13.6)	8.3 (18.8)	6.3 (18.7)	3.7 (16.2) **(n = 45)**

SD = standard deviation

fSRT (n = 63)	Enucleation (n = 41)	fSRT (n = 50)	Enucleation (n = 25)	fSRT (n = 41)	Enucleation (n = 20)	fSRT (n = 33)	Enucleation (n = 20)
1 year (form 4)		2 years (form 5)		3 years (form 6)		4 years (form 7)	
76.2 (13.6) **(n = 62)**	78.1 (19.0) **(n = 40)**	75.9 (15.7) **(n = 48)**	81.3 (15.0) **(n = 24)**	76.6 (18.9)	79.2 (15.9)	78.8 (14.8)	78.3 (12.8)
82. 6 (22.8) **(n = 62)**	80.9 (27.9)	86.7 (21.2) **(n = 48)**	84.0 (20.8)	84.2 (23.3)	81.0 (27.9)	84.3 (23.7) **(n = 32)**	91.6 (19.2) **(n = 19)**
83.1 (25.5) **(n = 62)**	75.6 (33.8)	83.7 (25.8) **(n = 49)**	79.2 (25.2) **(n = 24)**	85.4 (25.6)	82.5 (29.4)	90.6 (19.8) **(n = 32)**	79.0 (38.4) **(n = 19)**
81.2 (20.6) **(n = 62)**	79.5 (24.8)	80.8 (23.5) **(n = 48)**	83.7 (21.1) **(n = 24)**	84.8 (20.0)	80.4 (19.0)	83.3 (25.0)	84.6 (17.8)
80.1 (20.9) **(n = 62)**	84.2 (20.4)	84.7 (21.4) **(n = 48)**	86.8 (17.7) **(n = 24)**	81.7 (22.6)	83.3 (19.5)	80.8 (22.5)	90.0 (14.7)
92.2 (16.2) **(n = 62)**	91.1 (21.1)	89.6 (21.6) **(n = 48)**	92.4 (14.7) **(n = 24)**	88.2 (21.8)	92.5 (16.7)	86.9 (26.9)	91.7 (19.1)
27.0 (24.9) **(n = 62)**	27.1 (25.6)	24.5 (23.8) **(n = 49)**	18.0 (16.9)	24.9 (24.9)	27.2 (25.4)	28.3 (24.2)	18.7 (25.4) **(n = 19)**
3.2 (9.0) **(n = 62)**	8.5 (24.2)	5.1 (12.4) **(n = 49)**	2.0 (5.5)	3.3 (8.5)	3.3 (8.7)	1.5 (4.9)	4.4 (9.4) **(n = 19)**
19.9 (24.9) **(n = 62)**	19.1 (29.5)	19.1 (25.7) **(n = 49)**	16.0 (25.2)	18.7 (26.9)	15.0 (24.7)	21.2 (29.0)	10.8 (19.0)
11.5 (19.1) **(n = 61)**	8.1 (17.9)	8.8 (16.4) **(n = 49)**	4.0 (11.1)	9.8 (18.6)	11.7 (16.3)	11.1 (18.0)	10.5 (19.4) **(n = 19)**
19.7 (27.5) **(n = 61)**	21.1 (29.6)	23.1 (30.6) **(n = 49)**	24.0 (24.6)	13.8 (28.8)	21.7 (24.8)	14.1 (23.6)	21.1 (27.7) **(n = 19)**
5.4 (15.0) **(n = 62)**	13.8 (28.8)	4.1 (13.0) **(n = 49)**	2.8 (9.4) **(n = 24)**	5.7 (14.7)	6.7 (23.2)	4.0 (13.8)	10.5 (19.4) **(n = 19)**
4.8 (13.3) **(n = 62)**	9.8 (23.9)	2.8 (9.3) **(n = 48)**	6.9 (17.0) **(n = 24)**	5.7 (16.5)	10.0 (21.9)	4.0 (13.8)	5.0 (12.2)
4.9 (15.9) **(n = 61)**	5.7 (14.7)	3.6 (12.5) **(n = 47)**	6.9 (13.8) **(n = 24)**	6.5 (18.6)	3.3 (14.9)	3.1 (9.9) **(n = 32)**	6.7 (17.4)
3.8 (14.9) **(n = 62)**	6.5 (20.0)	6.9 (20.6) **(n = 48)**	2.8 (9.4) **(n = 24)**	5.7 (19.6)	1.7 (7.5)	8.1 (23.7)	5.0 (16.3)

DISCUSSION

In the subscales of the different questionnaires about the quality of life of UM patients treated either with fSRT or enucleation we noted dissimilarities. The most significant differences were that patients treated with fSRT improved in role functioning from 2 months until 4 years after treatment (p = 0.005; **Figure 2**) and fSRT patients reported better peripheral vision (p = 0.026; **Figure 3**) until 3 years after treatment compared to those that were enucleated. Role functioning is significantly (p = 0.012) decreased in patients treated with enucleation. This is comparable with Klingenstein *et al.*[28], who reported role physical as significant lower in the enucleation group versus stereotactic radiosurgery or control group. And this is in contrary with another study where role functioning increased significantly in patients treated with Ruthenium plaque or enucleation during the first year after therapy.[3]

In contrast to a previous report[3] regarding peripheral vision, we found that fSRT patients reported a better peripheral vision compared to enucleated patients (**Figure 3**). This is not surprising as the eye is conserved with the treatment. These results are in line with The Collaborative Ocular Melanoma Study (COMS), where patients treated with brachytherapy reported better peripheral vision for up to 2 years following treatment than enucleated patients.[29] Patients who underwent enucleation adapted to the new situation and got used to using their only remaining eye. Fifteen years after enucleation, 90% retained the ability to drive and 96% retained the ability to read.[30]

Cancer patients have reported disturbing sleeping patterns. Poor quality of sleep was correlated with poor quality of life.[31] In our study, patients with metastases reported a higher score in insomnia compared to the patients without metastases. However, this difference is only significant (p = 0.009, Student's t-test) 2 years after treatment. Other significant differences between those with and without metastatic disease were found for state anxiety, emotional functioning and appetite loss, 3 years after treatment. In addition, patients with metastases experienced more nausea 4 years after their treatment. Cancer-related anxiety may manifest as digestive symptoms, such as nausea.[32]

Overall we measured no significant differences in time effect in global health and in quality of life between both treatment groups. However, after four years we found a lower Global health score for patients with metastases (71) versus without metastases (78). Compared to other cancer patients, UM patients reported a substantial better quality of life score. The normative data for the EORTC QLQ-C30 in the general Dutch population shows an overall mean score of Global quality of life of 78 (SD: 17) for men (n = 935) and 77 (SD: 18) for women (n = 796).[33]

In this population the lowest global quality of life score was reported in patients with a self-reported depression in the past 12 months (mean: 60, SD: 20). The highest score was noted from people with no reported health problems (mean: 84, SD: 14). In our study, patients with UM reported a mean score of 76.4, which is almost comparable with the Dutch normative data and is higher than the scores of healthy people in Germany or the general population in Norway.[33-35] Chabert *et al.*[6] reported a decline of 5% in quality of life after treatment with either brachytherapy or radiotherapy. Unfortunately, the EORTC ophthalmic Oncology Quality of Life Questionnaire Module (EORTC QLQ-OPT30), an extra questionnaire module more specific for quality of life in uveal melanoma patients, was published 5 years after we started our study, and was not available at that moment.[18]

The DFS survival was significantly longer in patients treated with fSRT compared to enucleated patients ($p < 0.001$). However, when we adjusted for largest tumour diameter and tumour thickness, this finding was no longer significant. Enucleated patients had larger tumours and developed metastases more frequently than irradiated patients (**Table 1**). A larger tumour is related to a worse survival.[14] When we analysed the subgroup of patients with metastatic disease, after 1 year an increasing effect is reported with a Cohen's d factor of 0.85 after 4 years of treatment.

Despite the fact that this is not a randomized study, our results indicate that fSRT contributes to a better role functioning from 2 months posttreatment and improved peripheral vision from baseline until 36 months. However, in the Global health subscale, we found no difference in quality of life between the two treatment groups. The overall global health score, with no effect of time, was 76.4 (SD 13.6) on a scale of 0-100. If several treatment options are available for a patient, these results can aid in treatment planning.

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CHAPTER 10

DISCUSSION AND SUMMARY

CHAPTER 10.1

GENERAL DISCUSSION

GENERAL DISCUSSION

This thesis aims to contribute to the insight into the prognostic value of clinicopathological and chromosomal alterations and also aims to evaluate treatment outcomes of uveal melanoma (UM) patients. It provides a broad perspective on the complexity and the multifactorial aspects facing UM patients. Primary treatment of the tumour has shifted from enucleation to eye-sparing and although this approach is effective in obtaining local tumour control, it is not curative. Fifty percent of UM patients develop liver metastases despite successful eradication of the primary tumour. Unfortunately, most patients with metastases still die within a year in the absence of an adequate systemic treatment. Eventually, the goal is to diagnose and treat UM patients as quickly and effectively as possible and to provide them with the best quality of life. This chapter describes the most important findings in this thesis and places them in a broader perspective. Finally, suggestions for improvement of treatment strategies and future research will be provided.

Main findings and clinical relevance

Part I of this thesis focuses on the prognostic value of histopathologic characteristics and chromosomal alterations in UM. In the past decades, several clinical and histopathological risk factors have been identified and related to survival. A number of features of the primary tumour have been correlated with poor survival: these include epithelioid cell type, high mitotic activity, higher values of mean diameter of the ten largest nucleoli, higher microvascular density, presence of closed extracellular matrix patterns, tumour-infiltrating lymphocytes, tumour-infiltrating macrophages, higher expression of human leukocyte antigen Class I and Class II, largest basal tumour diameter, and extraocular extension (EXE).[1-3] Extraocular spread is possible through various transmission routes, such as aqueous drainage channels, ciliary arteries, vortex veins, ciliary nerves, the optic nerve, and a variety of rare combinations of these routes.[4] However, the route was not an important factor for systemic dissemination and tumour-related death. Tumours with EXE > 5 mm are classified in a subcategory with poor prognosis of the TNM (Tumour, Node, Metastases) classification of the American Joint Committee on Cancer (AJCC).[5] Extra ocular extension occurs in 2-15% of the UM patients.[1] We found that 12% of the patients had an EXE, and that an increase of 1 mm episcleral diameter led to a nearly 1.1 times increase in the risk of developing metastatic disease (Hazard ratio (HR): 1.08, 95% CI 1.01-1.15; p = 0.016). On the other hand, Coupland *et al.* [4] found no correlation with the size of the extraocular tumour (p= 0.3) and metastatic death. In addition, we observed that gain of chromosome 8q was associated with worse prognosis in patients with EXE, although this gain of 8q in relation to EXE had not previously been perceived to relate to survival. Indeed, chromosomal aberrations as loss of chromosome 3 (monosomy 3) and

gain of 8q seem to be even stronger predictors for worse prognosis than a higher staging of the AJCC classification or than those with either one of the aberrations or no aberrations at all.[6,7] The role of chromosome 3 and 8 is discussed further in this chapter. Clinical features of UM could be obtained through non-invasive measurements done by slit lamp examination or by ultrasound in the consultation room. Although genetic mutations are even stronger predictors, at the very least, all tumours should be staged following the TNM classification since every 1 mm increase in thickness of the tumour gives a decreased disease-free survival. In fact, even small melanoma could still have an unfavourable outcome.[8] Therefore, these small melanoma should ideally be treated as soon as possible before they metastasize or acquire genetic changes that predispose to metastasis. The risk for metastases could be best predicted if multiple clinical, genetic, and histologic factors are determined, such as has been done in the online tool to generate survival curves (http://www.ocularmelanomaonline.com).[7]

The TNM classification of UM is based on the extent of the primary tumour and on the presence of any systemic metastases. Lymph node involvement is extremely rare and only a few cases of UM patients with EXE with regional lymph node metastases have been described. The suggestion by Heindl *et al.* [9] that intraocular lymphangiogenesis in ciliary body melanomas with extraocular extension represents a new prognostic factor made us take a closer look at our EXE melanomas. Five immunohistochemical markers were used to differentiate between blood vessels and lymphatic vessels, and no evidence of the presence of intraocular lymphatic vessels could be found in UM with EXE. However, we did illustrate recruitment of subconjunctival lymphatic vessels into the EXE in a case of ciliary body melanoma. This might explain the regional lymphatic spread of UM cells to subconjunctival lymphatics through (iatrogenic) extrascleral spread of the tumour. Others sought to demonstrate lymphatic endothelium expression using only the following markers: D2-40 and CD34.[10] D2-40 and LYVE-1 are also expressed in lymphatic endothelium, in macrophages, and in other structures in the anterior segment of the eye as well. In addition, the vessels in UM or near UM could be focally positive for LYVE-1. False positive interpretation of the staining of the markers could be seen readily as proof for lymphatic vessels. This is why we suggested a panel of at least five immunohistochemical markers to detect lymphatic vessels, such as a positive staining for LYVE-1, D2-40, Prox-1, CD31 and a negative staining for CD34. Interestingly, the general idea is that orbital soft tissue does not contain lymphatic vessels, except for lymphatic-like structures around the optic nerve and lacrimal gland. We studied the development or presence of orbital lymphatic vessels during fetal and neonatal evolution and in adulthood. Positive staining for LYVE-1, D2-40, Prox-1, CD31 in combination with weak or absence of staining for CD34 was seen in 44% of the fetuses in a gestational age ranging from 13 to 24 weeks. No retrobulbar intraorbital lymphatic vessels were

observed in adult orbital tissue. Transient expression of lymphatic markers in retrobulbar orbital blood vessels and other tissues during fetal and early neonatal development has not been shown before. This could be valuable information for ocular pathologists who have to diagnose lymphatic malformations and underlines the assumption that there probably are no lymphatic vessels responsible for dissemination in UM patients.

As previously mentioned, many chromosomal risk factors for poor prognosis are well-established, and, recently, molecular genetic testing has been studied to refine metastatic risk. Metastases occurs mainly through a sequential, multi-step process.[11] While some patients already have metastases at the time of diagnosis, others develop metastases more than ten years after. To clarify this difference in development, the focus on UM research is now on copy number changes in chromosomes 1, 3, 6, 8, 9 and 11; mutations in *GNAQ*, *GNA11*, *EIF1AX*, *BAP1*; splicing factor mutations: *SF3B1*, *SRSF2*, *U2AF1* and gene expression profiling (GEP). UM have distinct copy number variation (CNV) patterns that correspond to different mutated driver genes.[12] Our results and those of other studies demonstrate the importance of loss of chromosome 3, gain of chromosome 8q, and being in a higher age group as an important prognostic factor for metastatic disease in UM.[13] UM without abnormalities in chromosomes 3 and 8 are associated with a 90% five-year survival whereas those with abnormalities in both these chromosomes indicate only a 45% five-year survival probability.[14] Of great importance is the *BAP1* gene, located on 3p21.31-p21; whereas monosomy 3 tumours are likely to metastasize with BAP 1 expression. Loss of *BAP1* protein expression or mutation of one allelic copy of *BAP1* without concomitant loss of chromosome 3 appears not to correlate with increased metastatic risk.[15]. The higher the percentage of monosomy 3 in tumour cells, the more aggressive the tumour is. We described a higher percentage (66-100%) of the cells with monosomy 3 correlates with even worse prognosis compared to other studies with monosomy 3 in general, such as a 4-year overall survival probability of 15-20% versus 30%, respectively.[16,17] In a multivariable analysis, we found that monosomy 3 (HR: 2.83, 95% CI 1.49- 5.39) had a lower prognostic value and longer disease-free survival than gain of 8q (HR: 3.13, 95% CI 1.54- 6.38). Additionally, a higher association with death has been found for gain of chromosome 8q (HR: 14.75) with a follow-up of five year after enucleation.[18] As the copy number of 8q tended to increase from primary tumours to metastasis, we observed that more CNV of 8q shortened survival.[19] In addition, poorer clinical outcome seems to be associated with higher chromosome 8q copy number and with a combination of monosomy 3 and gain of 8q.[1,13,20] Our study showed the importance of chromosome 3 and 8q in UM when taking prognostic or diagnostic biopsies to confirm UM diagnosis when in doubt. Further studies are needed to develop a clinically relevant prognostic model to identify patients with higher- versus lower-risk monosomy 3 UM. Prognostication could influence

the frequency of metastatic surveillance, prioritize high-risk patients for more aggressive adjuvant clinical trials, and provide information for patients to assist them in treatment choice.

Part II of this thesis focuses on treatment outcomes of UM and quality of life for UM patients. Historically, enucleation, removal of the eye, was the primary treatment for UM because it was thought that this could prevent the development of metastases. It seemed that at the time of diagnoses, tumour cells were already circulating in the peripheral blood and that metastases developed even years after enucleation. As a result of the large comparative studies of the COMS group (Collaborative Ocular melanoma Study group), the disease-free survival for radiotherapy and enucleation turned out to be more or less the same.[21] Enucleations were substituted for eye-sparing therapies, such as radiotherapy (i.e., brachytherapy, proton beam radiotherapy (PBR), (fractionated) stereotactic radiotherapy ((f)SRT)), photodynamic therapy (PDT), and surgical resection (i.e. endoresection or exoresection) of the tumour (**Table 1** in Introduction). Tumour necrosis can develop after radiotherapy, and this, in turn, leads to secretion of angiogenic factors, release of inflammatory stimuli, and retinal ischemia.[22]

A summary of 36 studies with 11.435 patients showed that radiotherapy has a local treatment failure rate ranging from 4.0-9.6%, with follow-up ranging from 20-150 months.[23-25] In a large cohort, 99% (95% CI 99-99) were no local recurrence was at 1 year, 93% (95% CI 92-94) at 5 years, and 89% (95% CI 86-91) at 10 years.[26] The downside of radiotherapy is ocular morbidity with visual loss and secondary enucleations due to tumour recurrence and untreatable neovascular glaucoma. We looked into our cohort of UM patients treated with fSRT and examined the radiation side-effects with at least a follow-up of five years. The most common side-effects were cataracts followed by retinopathy, maculopathy, vitreous haemorrhage (VH), neovascular glaucoma, and optic neuropathy. The median time to develop a side-effect ranges between 12.1 and 29.3 months. The adverse side-effects or complications does not differ from other radiation treatments for uveal melanoma.[27-29] By studying our treatment outcome, we should be able to improve the primary treatment and predict better when an adverse side-effect can occur. Since intravitreal injections of anti-vascular endothelial growth factors (VEGF) have become available, fewer side effects, such as radiation-induced retinopathy, maculopathy, VH, and neovascular glaucoma will develop due to earlier detection and treatment.[30] In case of neovascular glaucoma, one week after injection a regression of the iris and angle neovascularisation was noticed.[31] Unfortunately, retreatment remains necessary every few months. Especially since the first signs of glaucoma were seen after a median of 16-18 months, long term follow-up screening and monitoring seems necessary.[32] A side effect, such as maculopathy and subretinal fluid, could be easily detected since

the introduction of the non-invasive Optical Coherence tomography (OCT) scan. In eyes with radiation maculopathy discrete intraretinal hyperreflective spots have been documented on the spectral domain OCT.[33] These spots may be considered as a clinical biomarker of intraretinal inflammation in macular edema. In addition, enhanced depth imaging OCT (EDI-OCT) has been used to evaluate UM and has been shown to be helpful in detecting small UM and distinguishing it from small choroidal naevi and choroidal metastases.[34] UMs usually show regular surfaces on OCT and choroidal metastases have irregular and lobulated surface.[35]

There could be numerous reasons for a person to choose one treatment over another. To compare different radiotherapy treatments with enucleation would be unethical in a randomized control trial (the golden standard to compare treatments) for UM patients. To our knowledge, we were the first to compare the side-effects of radiation therapy in a matched dataset from UM patients treated with fSRT in Rotterdam, The Netherlands along with PBR in Liverpool, The United Kingdom. This provides the closest information to compare two treatment modalities, which have overlapping indications for treatment of UM. Matching was based on the following variables: age, gender, TNM-classification, tumour distances to the fovea and to the optic disc. In the matched dataset, the incidence of a VH was significantly higher after fSRT than after PBR (HR: 0.19; 95% CI 0.07-0.56; $p < 0.001$). Although VH at baseline and VH due to neovascular glaucoma were excluded, we were unable to explain why VH was more common after fSRT than after PBR. A VH is not a serious complication as it could easily be treated with vitrectomy, but patients would need to come to the hospital more often than if VH had not occurred. Tumour necrosis, proliferative radiation retinopathy, and posterior vitreous detachment have been suggested as presumed aetiology for VH.[36] For the other side effects, we found no differences after treatment between fSRT and PBR. Especially the actuarial rates for tumour recurrence, 4.5% for fSRT and 6.1% for PBR, were not significantly different between the two treatments. It is important to be aware of radiation-induced side effects and to treat the adverse side-effects promptly and adequately.[37] For patients, one radiotherapy modality could be advantageous over another for various reasons, but with our study, we report no differences in tumour control and in most adverse side effects.

Recently, there has been increasing emphasis on the importance of quality of life (QoL) in cancer care. Factors influencing QoL include life expectancy, general health condition, social and psychological support, and ocular factors, such as the severity of the UM, visual impairment, condition of the fellow eye, changes in appearance, day-to-day functioning, ocular discomfort, and worry regarding disease recurrence.[38] We conducted a prospective study to compare the QoL in UM patients after fSRT (n = 65) or after enucleation (n = 48). Enucleated patients had more difficulty working or performing household tasks (Role functioning

(p = 0.012)) from two months to four years after treatment compared to irradiated patients. Additionally, enucleated patients had diminished peripheral vision (p < 0.001) until three years after treatment. The COMS also found a better peripheral vision in irradiated patients, which is as expected.[39] It seems that enucleated patients do eventually adapt to the new situation, since after three years this difference in peripheral vision was gone; moreover, even 15 years after enucleation, 90% retained the ability to drive and 96% retained the ability to read.[40] After patients were diagnosed with UM, a medium level of distress was measured, which was reduced to a low level six months after treatment. If an ocular complication after radiotherapy occurs, patients have to visit the outpatient centre more often and this reminds them of their cancer. In the long term, the benefit in the aspect of emotional well-being of radiotherapy over enucleation treatment does not become significant.[39] However, in several other studies, patients after radiotherapy (compared to enucleation) had less ocular discomfort, less visual difficulty, less worry about their appearance and about future poor health, their risk of metastatic disease, and their risk of losing their eye.[38,41] In our study, QoL was not significantly different between the two treatments. Recently, a large systematic review based on 18 studies analysed 4285 patient and found that no treatment modality provides for improved QoL outcomes.[42] Only 33% of the studies reported a significant difference in at least one QoL domain for one treatment modality compared with the other(s).[42]

As expected, all our UM patients with metastases experience more anxiety (p = 0.026) after three years, more insomnia after two years (p = 0.009), more appetite loss (p = 0.014) after three years, and more distress (p = 0.037) than patients without metastases. In a pilot study, higher anxiety scores were reported in patients with metastases shorter than 1 year and lower anxiety scores in patients with more than 5 years metastases.[43] Furthermore, chromosome 3 loss was associated with higher levels of anxiety.[41] The self-reported health-related quality of life test has a global health score on a scale of 0 to 100, with a high score indicating a good quality of life. The mean global health subscale score in our group was 76.4 (SD 13.6) and not significantly different between patients with metastases (71) and those without metastases (78). Compared to other cancer patients, UM patients report a substantial better quality of life. The mean score of 76.4 is almost comparable with the Dutch normative data of persons without cancer (mean scores: 77.4-78.0).[44,45]

The patient perspective is sometimes forgotten in considerations of how clinical cancer care could be improved. An online survey among UM patients in the United States showed that patients regret that there was a delay in diagnosis and one-third of the UM was first diagnosed as a naevus.[46] Patients complained that they did not know about prognostic biopsy and lacked psychological care. Cancer survivors should be monitored to prevent, detect, and intervene in the

development of anxiety and depression.[47] We are not there yet; nevertheless, ocular oncologists and researchers have achieved more insight into the genetic factors that are responsible for the development of metastatic disease in UM. Still, there is much unknown about those genes, and hopefully, some day in the near future, systemic therapy will be improved and will be able to prevent the development of metastases.

Future prospects

The first step to improve the patient's outcome is to diagnose a suspicious lesion or a lesion as an UM. Delay in treatment could have implications on preventable morbidity and patient outcome. The chance in Finland of being immediately referred and correctly diagnosed at the first visit was 89% and 71%, respectively.[48] In Britain, misdiagnosis occurred in 25% of the patients seen by an ophthalmologist.[49] Moreover, some of the UM patients had shortly before been seen by an ophthalmologist for unrelated reasons without the tumour being diagnosed. This implies that good education for optometrists, residents, and ophthalmologists is crucial and should focus on the features that promote the ability to recognize a suspicious lesion and to differentiate a choroidal naevus from a small melanoma.[50] Since smaller melanoma usually do have better prognosis, this education could extend patients' lives. Using imaging technology, such as OCT-scan, is another aid to early detection. The OCT-scan is more sensitive in detecting smaller changes of naevi and potential UM than ultrasonography. Even the suspicious features of naevi with subretinal fluid and orange pigment can be readily seen on OCT-scan[34]; the presence of orange pigment is autofluorescent on OCT-scan. It is important that patients with naevi should be told to go to an ophthalmologist if they have symptoms.

Most UM are treated with radiotherapy; this requires the ability to treat the vision threatening iatrogenic side-effects and complications. Currently, intravitreal anti-VEGF or steroid injections may prevent or delay radiation-induced macular edema. However, prospective studies analyzing early characteristics of radiation retinopathy are limited and would be helpful to distinguish between a high risk of macular edema and a low risk of macular edema. There is a need and importance of developing a standardized, complete assessment tool tailored to UM. Studies with matched data (i.e. brachytherapy versus PBR and brachytherapy versus fSRT) could provide precise information about ocular morbidity after the different radiotherapy treatment options. Standardization of study methods and outcome measures will allow better comparison of survival data derived from different prognostic tests.[51] Prognostication in UM has implications for patient counselling, follow-up, and for enrolment for clinical adjuvant therapy trials. Presence of monosomy 3, gain of chromosome 8q, and loss of BAP1 protein are

strong predictors for a decreased disease-free survival, whereas deletion of 6q or gain of 6p indicates a low risk. Germline *BAP1* mutated individuals with the BAP1 tumour genetic predisposition syndrome should have an annual ophthalmic exam by an ocular oncologist and other specialists starting at age 16, and biannual exams after age 30 is recommended. Exams should even be considered earlier if there is family history of UM.[52]

Prognostic biopsy of the tumour helps with the detection of chromosomal or mutational defects and may soon become the standard of care. Targeted next generation sequencing of UM obtains mutation and copy number variation data and can predict patients' outcome.[53] However, the heterogeneity of the tumour should be kept in mind.[54] Moreover, there are also a small number of patients with 'favourable' genetic risk factors who still develop metastases. On the other hand, since tumour tissue is not always available and biopsies are not without risk, there is an ongoing search for non-invasive predictors of metastatic disease. These 'liquid biopsies' provide tumour cells and derived molecules from blood. The accuracy of these non-invasive techniques will increase and will therefore give a better understanding about the genetic and epigenetic mechanisms that contribute to metastases. The development of metastases in UM is associated with changes in immune effector and regulatory cells consistent with lessening tumour immune surveillance.[55,56] These changes are related with changes in plasma and cellular levels of immune regulatory microRNAs (miRNAs). Four main miRNA clusters were associated with monosomy 3 and its DNA methylation state, and even more miRNAs are being detected every week.[57] Other research about extracellular vesicles (exosomes) from cultured UM cells, circulating tumour cells (CTCs), and circulating DNA (ctDNA) are being investigated extensively. These biomarkers could provide more information about the tumour load and the metastatic risk and, hopefully, will give targeted systemic therapy in the future.
Because important genes responsible for UM have been found in recent decades, the understanding of the development of UM and metastases at the cellular level is still unclear. Several promising new agents in clinical trials are in development for UM. Hence, this gives hope that new and more effective treatment options will become available in the near future for UM patients with metastatic disease.

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CHAPTER 10.2

SUMMARY

SUMMARY

Although uveal melanoma (UM) are rare ocular tumours, they are, nevertheless, associated with significant morbidity and mortality. In about half of UM patients, the cancer will eventually metastasize, usually to the liver. The disease-free survival varies between low-risk versus high-risk metastatic profile groups. Unfortunately, most patients with metastases still die within a year in the absence of an adequate systemic treatment. The development of UM and metastases is a complex multi-factorial process whose basic mechanism still remains unclear. Clinical factors, histopathological parameters, chromosomal aberrations, mutations of genes, epigenetics, and gene expression profiles can all have an effect on the disease-free survival. Optimizing cancer treatment based on prognostic factors plays a crucial role in the management of UM. In the first part of this thesis, we describe the prognostic value of clinical and histopathologic characteristics, locoregional anatomic factors, and chromosomal alterations in UM. The second part of this thesis illustrates the treatment outcome and quality of life of patients with UM. Finally, we summarize our main findings and provide a general discussion and assess future prospects.

Chapter 1 provides a general introduction to UM and describes the aims of this thesis. Subsequently, **part I** of this thesis is based on the prognostic value of the histopathological characteristics and chromosomal alterations in UM discussed in chapters 2 to 6. Extra ocular extension (EXE), growth of the tumour outside the sclera, occurs in 2-15% of UM. **Chapter 2** discusses the prognostic value of EXE in relation to monosomy 3 (loss of chromosome 3) and gain of chromosome 8q. Although studied in a small group, a larger episcleral diameter of the EXE and an additional copies of chromosome 8q correlates with a worse prognosis than a normal chromosome 8q content. Moreover, we found that disease-free survival or metastasis-free survival is significantly reduced in UM patients with the following tumour characteristics: large basal tumour diameter, extracellular matrix patterns, monosomy 3, and gain of chromosome 8q. Extraocular extension is an important prognostic factor, as patients with EXE developed metastases or died due to metastases almost 6 years earlier on average, compared to patients without EXE. This work confirms the useful addition of EXE to the TNM (Tumour Node Metastases) Classification of malignant melanoma of the uvea, published in 2017. In **Chapter 3**, we propose a panel of antibodies to detect intraocular lymphatic vessels with high specificity. Only when the Lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1), Podoplanin (D2-40), Prospero-related homeobox gene-1 (Prox-1), and pan-endothelial marker CD31 show positive staining combined with the negative expression of blood vessel endothelium specific CD34, can a vascular structure be classified as a lymphatic vessel. We observed that intraocular lymphatic vessels are absent in UM with EXE; however, we provide proof for recruitment of intratumoural

lymphatic vascular structure in the periphery of the subconjunctival EXE. This may explain the rare cases of regional lymphatic spread of UM. In addition, the expression of lymphatic markers is examined in the retrobulbar intraconal orbit of the developing and the adult eye in **Chapter 4**. We emphasized the maturation dependent changes and observed transient expression of lymphatic markers in the retrobulbar intraconal orbital vasculature during fetal and early neonatal development. No expression of the five lymphatic markers was detected in the adult orbital vasculature. Hence, we concluded that the orbit can be envisioned to possess a full range of lymphatic plasticity. In **Chapter 5**, tumour cells were divided according to the FISH (fluorescence *in situ* hybridization) count in three groups with increasing percentages of loss of chromosome 3 or gain of chromosome 8q and were related to disease-free survival. It appeared that monosomy 3, gain of chromosome 8q, and older age were the most important independent prognostic factors for worse survival. Additionally, a higher percentage of monosomy 3 and a gain of chromosome 8q in tumour cells showed a strong relation with worse disease-free survival compared to lower percentages of these aberrations. Interestingly, an increased number of additional copies of 8q correlated with shorter survival. This study emphasizes the importance of gaining chromosome 8q as a cytogenetic factor for metastatic disease in UM. In **Chapter 6**, we describe an UM patient who developed liver and pancreatic metastases 8 years after primary treatment of UM. Chromosomal aberrations and targeted gene mutations were analyzed in the tumour DNA, isolated from the metastatic tissue and the primary UM. The heterozygous early *GNA11* mutation together with the *SF3B1* mutation were observed in all the samples. The *SF3B1* mutation seems to predispose for late metastatic disease in the absence of a *BAP1* mutation. Typical chromosomal aberrations for a *SF3B1* profile were observed, such as loss of chromosome 1 p, gain of chromosome 6p, and gain of chromosome 8q.

In **part II** of this thesis, we provide an overview of the clinical factors influencing treatment outcome and the quality of life of UM patients after treatment. **Chapter 7** describes the local tumour control, disease-free survival, visual preservation. and radiation side effects in the Erasmus MC cohort of UM patients treated with fractionated stereotactic radiotherapy (fSRT) with at least a 5-year follow-up. The 1, 5, 10 and 15 years cumulative local tumour control rates were: 99.4%, 92.2%, 89.3% and 89.3%, respectively. When the tumour recurs, significantly more enucleations (removal of the eye) is required. We also observed that larger tumours were an independent risk factor for worse disease-free survival. The cumulative incidence of disease-free survival at 1, 5, 10, 15 years were 96.3%, 77.0%, 69.9% and 61.9%, respectively. The visual outcome decreased significantly after treatment from 0.15 logMAR at time of diagnosis to 0.49 logMAR after 12 months; where a higher logMAR indicates a worse visual acuity. This lower visual outcome was mainly due to the most common side effects of fSRT, such as cataract (67.8%),

followed by retinopathy (35.1%), maculopathy (23.8%), vitreous haemorrhage (20.1%), neovascular glaucoma (20.0%), and optic neuropathy (12.4%). In this study population, we found that anterior located UM developed cataract more often after treatment. There is an overlap in treatment indications, tumour control, and radiation-induced adverse effects between fSRT and other treatment modalities, such as proton beam radiotherapy (PBR). Treatment outcomes of fSRT and PBR have been extensively evaluated independently; however, as presented in **Chapter 8**, we were the first to our knowledge to describe ocular outcomes of fSRT and PBR in matched data groups of Rotterdam and Liverpool, the United Kingdom, respectively. The UM patients were matched according to sex, the left or right eye, the Tumour (T) category using the 8th edition of the AJCC (American Joint Committee on Cancer) Cancer Staging Classification, and the location of the UM – posterior margin ≤ or > 3 mm of the fovea and of the optic disc. Both treatments revealed excellent tumour control; the 5-year and 10-year actuarial rates were 4.5% and 9.8% for fSRT and 6.1% and 11.9% for PBR. Of the adverse effects after radiotherapy, only vitreous heamorrhage was more common after fSRT than after PBR. In general, larger UMs were a risk factor for developing maculopathy and secondary enucleation independent of the treatment modality. In **Chapter 9**, we report quality of life and visual functioning of UM patients treated with enucleation or fSRT in a prospective study. Enucleated patients had more difficulty working or performing household tasks for the first 2 months compared to fSRT patients. The first 3 years after enucleation, patients complained of less peripheral vision compared to fSRT patients. Moreover, having metastatic disease revealed more subjective distress than without metastatic disease. Although all patients suffered from insomnia, anxiety, and reduced physical functioning, the quality of life is not significantly different between the two treatment groups.

In **Chapter 10**, we discuss our main findings and provide an interpretation of these findings. And finally, we discuss the future prospects. The studies in this thesis not only emphasize the complexity of all factors influencing prognosis, but also provide an overview of the considerations when choosing a treatment modality.

CHAPTER 10.3

NEDERLANDSE SAMENVATTING

SAMENVATTING

Uveamelanomen (UM) zijn zeldzame oculaire tumoren, die geassocieerd zijn met een significante morbiditeit en mortaliteit. Bij ongeveer de helft van de patiënten zal de kanker uiteindelijk metastaseren, meestal naar de lever. Tussen de groepen met een laag en hoog risico op metastasen varieert de ziektevrije overleving op basis van een verschillend profiel. Helaas sterven de meeste patiënten met metastasen nog steeds binnen een jaar vanwege een gebrek aan een adequate systemische behandeling. De ontwikkeling van UM en metastasen is een complex multifactorieel proces, dat nog steeds niet is opgehelderd. Klinische factoren, histopathologische parameters, chromosomale afwijkingen, mutaties van genen, epigenetische factoren en genexpressie profielen kunnen allemaal effect hebben op de duur van de ziektevrije overleving. Het optimaliseren van de behandeling van kanker op basis van prognostische factoren speelt een cruciale rol bij uveamelanomen.

Het eerste deel van dit proefschrift geeft de prognostische waarde van klinische en histopathologische kenmerken, locoregionale anatomische factoren en chromosomale afwijkingen van UM weer. Het tweede deel van dit proefschrift illustreert het resultaat van de behandeling en de kwaliteit van leven van uveamelanoom patiënten. Tot slot worden de belangrijkste bevindingen samengevat en bediscussieerd en worden overwegingen en ideeën voor toekomstig onderzoek beschreven.

Hoofdstuk 1 geeft een algemene inleiding over UM en beschrijft het doel van dit proefschrift. Vervolgens is **deel I** van dit proefschrift**,** de hoofdstukken 2 tot en met 6, gebaseerd op de prognostische waarde van histopathologische kenmerken en chromosomale afwijkingen in UM. Extra oculaire extensie (EXE), groei van de tumor buiten de sclera, treedt op in 2-15% van de UM. **Hoofdstuk 2** behandelt de prognostische waarde van EXE in relatie tot monosomie 3 (verlies van chromosoom 3) en winst van chromosoom 8q. Hoewel dit in een kleine groep tumoren is onderzocht, correleert een grotere episclerale diameter van de EXE en daarbij winst van 8q met een slechtere prognose dan een normaal aantal kopieën van chromosoom 8q. Daarbij was de ziektevrije overleving of de metastase-vrije overleving aanzienlijk verminderd in uveamelanoom patiënten met EXE en de volgende tumor kenmerken: een grote basale tumordiameter, extracellulaire matrixpatronen, monosomie 3 en winst van chromosoom 8q. Extraoculaire uitbreiding is een belangrijke voorspellende factor, omdat patiënten met EXE gemiddeld bijna 6 jaar eerder metastasen ontwikkelden of stierven als gevolg van metastasen, vergeleken met patiënten zonder EXE. Deze studie bevestigt de waardevolle toevoeging van EXE aan de TNM (Tumor Node (Lymfklier) Metastase) classificatie van uveamelanomen gepubliceerd in 2017. In **hoofdstuk 3** wordt een panel van antilichamen voorgesteld om intraoculaire lymfevaten met een hoge specificiteit te detecteren. Alleen wanneer de markers

LYVE-1 (Lymphatic vessel endothelial hyaluronic acid receptor-1), D2-40 (Podoplanin), Prox-1 (Prospero-related homeobox gene-1) en CD31 (pan-endothelial marker) een positieve aankleuring vertonen in combinatie met een afwezige expressie van CD34 (blood vessel endothelium specific marker) kan een vasculaire structuur worden geclassificeerd als een lymfevat. De intra-oculaire lymfevaten bleken afwezig te zijn in UM met EXE; echter, we leveren bewijs voor de rekrutering van intra-tumorale lymfevaten in de periferie van de subconjunctivale EXE. Dit zou de zeldzame gevallen van lokale lymfklier metastasen bij UM kunnen verklaren. Daarnaast wordt de expressie van lymfemarkers onderzocht in de retrobulbaire-intraconale orbita van het ontwikkelende en volwassen oog in **hoofdstuk 4**. We hebben de nadruk gelegd op de veranderingen die afhankelijk zijn van de ontwikkeling en de tijdelijke expressie van lymfvatenmarkers in de retrobulbaire-intraconale vaten tijdens de foetale en vroege neonatale ontwikkeling. Er werd geen enkele expressie van de vijf lymfvatenmarkers gedetecteerd in de volwassen vaten. De conclusie die hieruit volgde was dat de orbita in ontwikkeling in staat is tot een volledig scala aan lymfeplasticiteit. In **hoofdstuk 5** werden tumorcellen verdeeld in drie groepen middels FISH (fluorescentie *in situ* hybridisatie) techniek met een toenemend percentage van het verlies van chromosoom 3 of van de winst van chromosoom 8q en deze groepen werden onderzocht naar de ziektevrije overleving. Monosomie 3, de winst van chromosoom 8q en oudere leeftijd waren de belangrijkste onafhankelijke voorspellende factoren voor een slechtere overleving. Bovendien toonden hogere percentages monosomie 3 en winst van chromosoom 8q in tumorcellen een sterkere relatie met een slechtere ziektevrije overleving in vergelijking met lagere percentages van deze chromosomale afwijkingen. Interessant is dat een groter aantal extra kopieën van 8q gecorreleerd is met een kortere overleving. Deze studie benadrukt het belang van extra materiaal van chromosoom 8q als cytogenetische factor voor de ontwikkeling van metastasen van UM. In **hoofdstuk 6** beschrijven we een uveamelanoom patiënt met lever- en pancreas metastasen, die 8 jaar na de primaire behandeling van zijn melanoom metastasen ontwikkelde. Chromosomale afwijkingen en gen mutaties werden geanalyseerd in het tumor DNA vanuit het geïsoleerd weefsel van metastasen en de primaire tumor. In al deze weefsels vonden we een heterozygote *GNA11* mutatie samen met de bekende R625 *SF3B1* mutatie. De *SF3B1* mutatie lijkt, bij afwezigheid van een *BAP1* mutatie, bepalend te zijn voor een late ontwikkeling van metastasen. Typische chromosomale afwijkingen werden gevonden passend bij een *SF3B1* mutatie profiel, zoals het verlies van chromosoom 1 p, winst van chromosoom 6p en winst van chromosoom 8q.

In **deel II** van dit proefschrift wordt een overzicht gegeven van de klinische factoren die de uitkomst van de behandeling en de kwaliteit van leven van uveamelanoom patiënten na behandeling beïnvloeden. **Hoofdstuk 7** beschrijft de lokale tumorcontrole, ziektevrije overleving, visus of gezichtsscherpte en de bestralingsbijwerkingen van uveamelanoom patiënten na behandeling met

gefractioneerde stereotactische radiotherapie (fSRT) in het Erasmus MC, met een follow-up van ten minste 5 jaar. De cumulatieve lokale tumorcontrole van 1, 5, 10 en 15 jaar bedroeg respectievelijk 99.4%, 92.2%, 89.3% en 89.3%. Wanneer de tumor recidiveert of groeit, is een enucleatie (verwijdering van het oog) nodig. Een grotere tumor was een onafhankelijke risicofactor voor een slechtere ziektevrije overleving. De cumulatieve incidentie van ziektevrij overleven op 1, 5, 10, 15 jaar bedroeg respectievelijk 96.3%, 77.0%, 69.9% en 61.9%. De visus daalde aanzienlijk na behandeling van 0.15 logMAR op het moment van diagnose stellen naar 0.49 logMAR na 12 maanden na behandeling; waarbij een hogere logMAR duidt op een slechtere gezichtsscherpte. Deze lagere visus was voornamelijk te wijten aan de meest voorkomende bijwerkingen van fSRT, zoals cataract (67.8%), gevolgd door retinopathie (35.1%), maculopathie (23.8%), glasvocht bloedingen (20.1%), neovasculair glaucoom (20.0%) en opticopathie (12.4%). In de onderzoekspopulatie bleek datde ogen met tumoren voorin het oog vaker cataract als bijwerking ontwikkelden dan de tumoren achterin het oog. Er is een overlap in behandelingsindicaties, tumorcontrole en bijwerkingen tussen fSRT en een andere behandeloptie, zoals protonen bestraling (PBR). De resultaten van de behandeling van fSRT en PBR zijn onafhankelijk uitvoerig geëvalueerd, maar, zoals gepresenteerd in **hoofdstuk 8**, menen we de eerste te zijn die de resultaten van fSRT en PBR beschrijft in studiegroepen, van Rotterdam en Liverpool, die werden gematched. De uveamelanoom patiënten groepen werden vergeleken en gekoppeld op basis van geslacht, linker- of rechteroog, Tumor (T)- categorie van de 8^{e} editie van de AJCC (American Joint Committee on Cancer) Cancer Staging Classification, tumor locatie: achterste grens van de tumor ≤ of > 3 mm van de fovea en van de nervus opticus (oogzenuw). Beide behandelingen lieten een uitstekende tumorcontrole zien; de 5 en 10 jaar lokale tumor controle bedroegen 4.5% en 9.8% voor fSRT en 6.1% en 11.9% voor PBR. Van alle bijwerkingen na radiotherapie kwam alleen een glasvochtbloeding vaker voor na fSRT dan na PBR. In de gehele populatie was, onafhankelijk van de behandeling, een grotere afmeting van de tumor een risicofactor voor de ontwikkeling van maculopathie en secundaire enucleatie. In **hoofdstuk 9** wordt de kwaliteit van leven en de visuele uitkomst van patiënten gerapporteerd, die in een prospectief onderzoek behandeld zijn middels enucleatie of fSRT. Geënucleerde patiënten hadden de eerste 2 maanden meer moeite met het werken of uitvoeren van huishoudelijke taken dan patiënten na fSRT. De eerste 3 jaar na enucleatie klaagden patiënten over minder perifeer zicht dan patiënten na fSRT. Bovendien bleek het hebben van metastasen meer angst te geven dan het hebben van geen metastasen. Hoewel alle patiënten slapeloosheid, angst en vermindering van het fysieke functioneren ervaren, is de kwaliteit van leven niet significant verschillend tussen de beide behandelingsgroepen.

In **hoofdstuk 10** worden de belangrijkste bevindingen besproken en geïnterpreteerd. Tot slot worden de vooruitzichten en ideeën besproken voor toekomstig onderzoek. De studies in dit proefschrift benadrukken niet alleen de complexiteit van alle factoren die de prognose beïnvloeden, maar geven ook een overzicht van de overwegingen bij het kiezen van een behandeling voor uveamelanoom patiënten.

CHAPTER 11

APPENDICES

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ALT	alanine transaminase
AST	aspartate aminotransferase
BAP1	BRCA1-associated protein-1
BAP1-TPDS	BAP1 tumour predisposition syndrome
BCVA	best corrected visual acuity
CD	cluster of differentiation
CD11b+	cluster of differentiation 11b (monocytes/macrophages)
CD-31	Platelet endothelial cell adhesion molecule (PECAM-1) also known as cluster of differentiation 31
CD-34	transmembrane phosphoglycoprotein protein encoded by the cluster of differentiation 34 gene
CGH	comparative genomic hybridization
CHRPE	congenital hypertrophy of the retinal pigment epithelium
CI	confidence interval
CME	cystoid macular edema
CNV	copy number variation
COMS	Collaborative Ocular Melanoma Study
CORR	Collaborative Ophthalmic Research Rotterdam
CT	computed tomography
CTCs	circulating tumour cells
ctDNA	circulating DNA
CYSLTR2	cysteinyl leukotriene receptor 2
D2-40	monoclonal antibody against Podoplanin
DFS	disease-free survival
DNA	deoxyribonucleic acid
EDI-OCT	enhanced depth imaging optical coherence tomography
EIF1AX	eukaryotic translation initiation factor 1A, X-linked
EORTC QLQ-C30	European Organization for Research and Treatment of Cancer Quality of Life Questionnaire
EORTC QLQ-OPT30	European Organization for Research and Treatment of Cancer ophthalmic oncology quality of life module
EXE	extraocular extension
FAMM	familial atypical mole and melanoma syndrome
FFPE	formalin-fixed and paraffin-embedded
FISH	fluorescent in situ hybridization
Flt-4	Fms related tyrosine kinase 4
fSRT	fractionated stereotactic radiotherapy
GEP	gene expression profiling
GNA11	guanosine nucleotide-binding protein alpha-11
GNAQ	guanosine nucleotide-binding protein Q polypeptide
Gy	Gray, unit of ionizing radiation dose
H&E	haematoxylin and eosin
HGF	hepatocyte growth factor
HR	Hazard ratio
IES	impact of event scale

IGF-1	insulin-like growth factor 1
IQR	interquartile range
Kbp	kilobase pairs
LDH	lactate dehydrogenase
LINAC	linear particle accelerator
lncRNA	long non-coding RNAs
logMAR	Logarithm of the Minimum Angle of Resolution
LOH	loss of heterozygosity
LTD	largest tumour diameter
LYVE-1	lymphatic vessel endothelial hyaluronan receptor type 1
MAPK	mitogen-activated protein kinase
MAQ	multiplex amplicon quantification
MFS	metastasis-free survival
MIA	melanoma inhibitory activity
miRNA	microRNAs
MLPA	multiplex ligation probe amplification
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSA	microsatellite analysis
NEI VFQ-25	national eye institute visual function questionnaire
NF1	neurofibromatosis type 1
NGS	Next Generation Sequencing
NVG	neovascular glaucoma
OCT	optical coherence tomography
OPN	osteopontin
PA	phosphatidic acid
PAS	Periodic-acid Schiff
PBR	Proton beam radiotherapy
PDT	photodynamic therapy
PECAM-1	Platelet endothelial cell adhesion molecule-1
PEHC	peripheral exudative hemorrhagic chorioretinopathy
PKC	protein kinase C
PLCB4	Phospholipase C Beta 4
Prox-1	Prospero-related homeobox gene-1
QOL	quality of life
qPCR	quantitative polymerase chain reaction
ROMS	Rotterdam Ocular Melanoma Studygroup
RPE	retina pigment epithelia
Ru-106	ruthenium-106
SD- OCT	spectral domain optical coherence tomography
SD	standard deviation
SF3B1	splicing factor 3B subunit 1
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
SRSF2	serine- and arginine-rich splicing factor 2
SRT	stereotactic radiotherapy
STAI	state-trait anxiety inventory

TAZ	transcriptional coactivator with PDZ-binding motif
TGCA	The Cancer Genome Atlas
TNM	Tumour Node Metastases
TPS	tissue polypeptide specific antigen
TTT	trans pupillary thermotherapy
U2AF1	U2 small nuclear RNA auxiliary factor
UM	uveal melanoma
US	ultrasonography
VEGF	vascular endothelial growth factor
VEGF-C	vascular endothelial growth factor-C
VH	vitreous haemorrhage
YAP	Hippo-Yes-associated protein
γGT	gamma-glutamyl transpeptidase

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PHD PORTFOLIO

Name PhD student:	Jackelien van Beek
Erasmus MC Department:	Ophthalmology Medisch Genetisch Centrum zuid-west Nederland (MGC)
PhD period:	2011-2021
Promotor:	Dr. J.E.M.M. de Klein
Copromotors:	Dr. E. Kiliç and Dr. R.M. Verdijk

1. PhD training

	Year	Workload (Hours/ ECTS*)
Courses		
Teach the Teacher II: Two-day training Basic didactics for supervisors of interns	2011	16 hours
Research Integrity	2015	0.3 ECTS
Statistics: Survival analysis, NIHES EWP24	2016	1.9 ECTS
Biomedical English Writing and Communication	2018	4.0 ECTS
Specific courses		
IELTS exam and preparation course with English tutor	2012	30 hours
Residency Ophthalmology, Erasmus MC, Rotterdam	2012	23 months
Seminars and workshops		
Diabetic Macular Edema Masterclass	2014	2 hours
Maastricht Ultrasound Course	2016	5 hours
'Donders gezelschap voor strabologie', Dordrecht	2016	6 hours
Retina symposium: 'Setting new standards in retinal disease care', Amsterdam	2017	4 hours
Nineteenth Glaucoma Symposium, Rotterdam	2017	4 hours
Update on eyes, Seefeld, Austria	2017	10 hours
Update on eyes, Seefeld, Austria	2019	10 hours
'Rode Hoed Symposium- De Medisch specialist', Amsterdam	2019	5 hours

	Year	Workload (Hours/ ECTS*)
Presentations		
(Inter) national conferences		
NOG Annual Meeting 2011, Maastricht (oral presentation)	2011	30 hours (1.1)
NOG Annual Meeting 2012, Groningen (oral presentation)	2012	30 hours (1.1)
NOG Annual Meeting 2013, Groningen	2013	18 hours
ARVO Annual Meeting 2013, Seattle, USA (poster presentation)	2013	40 hours (1.4)
ESCRS Annual Meeting 2013, Amsterdam	2013	40 hours (1.4)
OOG, Annual Meeting 2014, Cracow, Poland, (oral presentation),	2014	40 hours (1.4)
NOG Annual Meeting 2014, Maastricht (oral presentation)	2014	30 hours (1.1)
NIOIC and NGRC symposium, Rotterdam	2014	8 hours
NOG Annual Meeting 2015, Groningen (oral presentation)	2015	30 hours (1.1)
OOG, Annual Meeting 2015, Moscow, Russia (poster presentation)	2015	40 hours (1.4)
EURETINA, 6th Winter meeting of European society of retina specialists	2016	8 hours
OOG, Annual Meeting 2016, Athens, Greece	2016	40 hours (1.4)
OOG, Annual Meeting 2017, Rotterdam	2017	40 hours (1.4)
NOG Annual Meeting 2017, Maastricht (poster presentation)	2017	30 hours (1.1)
EVER/OOG Annual Meeting 2018, Nice, France (oral presentation)	2018	40 hours (1.4)
NOG Annual Meeting 2019, Maastricht	2019	18 hours
NOG Annual Meeting 2020, Virtual Conference (presentation)	2020	12 hours

2. Teaching

	Year	Workload (Hours/ ECTS*)
Lecturing		
Weekly scientific seminars at the department of Ophthalmology, Erasmus MC, Rotterdam (oral presentations)	2011-2013	80 hours
Lecturing medical students	2011-2013	40 hours
Lecturing interns	2011-today	720 hours
Pathology meeting, Erasmus MC, Rotterdam, Thursdays monthly	2011-2013	30 hours
OCOO and lecturing, Albert Schweitzer Hospital, Dordrecht (oral presentations)	2015 2018	3 hours 4 hours
Teaching, tutoring and supervising Master's theses		
Co-supervising M. Dallinga, 6th year medical student	2011-2012	6 months
Co-supervising Q. van den Bosch, MSc	2018	2 months
Supervising M. Bergman, ANIOS Ophthalmology	2019	3 months

* 1 ECTS (European Credit Transfer System) equals a workload of 28 hours.

ABOUT THE AUTHOR

Jackelien van Beek was born on December third 1979 in Nijmegen, the Netherlands. She graduated from the secondary school in 1998 and applied for the study Medicine. Unfortunately, due to numerous fixus, she started the same year her second-choice study: Health Sciences at Maastricht University in Maastricht. After two years, she transferred to the Erasmus University Rotterdam to study Medicine. During her study she worked as a medical student at the department of Epidemiology and Neurosurgery at the Erasmus MC. Since the start of the study Medicine, she participated for two years at the national rowing team at Skadi, Rotterdam. In 2004 she was selected to start a research project about arterial ischemic stroke in children under the supervision of Prof. dr. A.I.R. Maas and Prof. M.V. Johnston for 6 months at Johns Hopkins University Hospital, Baltimore, USA. After obtaining her medical degree in 2006, she started her residency in Ophthalmology at the department of Ophthalmology at Erasmus Medical Center, headed by Prof. dr. G. van Rij and later Prof. dr. J.R. Vingerling. For the last internship she spends one month in Ambon Hospital, Ambon, Indonesia to select patients for cataract surgery under the supervision of F. Tegelberg, ophthalmologist. During the last two years of her residency, she started her PhD project in 2011. In 2013 she became an ophthalmologist and worked in a clinic and several hospitals. Currently, she is working as an ophthalmologist at the Ikazia Ziekenhuis and Eye Clinic 'Oog op Zuid' in Rotterdam. Jackelien lives with Sander van den Heuvel and their two children Simon (2012) and Philine (2015) in Rotterdam.

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Jackelien