OCULAR MELANOMA

Insights into genetics, inheritance
and testing

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Ocular Melanoma

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General introduction

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ABSTRACT

Ocular melanoma consists of posterior uveal melanoma, iris melanoma and conjunctival melanoma. These malignancies derive from melanocytes in the uveal tract or conjunctiva. The genetic profiles of these different entities differ from each other. In uveal melanoma, *GNAQ* and *GNA11* gene mutations are frequently found and prognosis is based on mutation status of *BAP1*, *SF3B1* and *EIF1AX* genes. Iris melanoma, also originating from the uvea, has similarities to the genetic makeups of both posterior uveal melanoma (UM) and conjunctival melanoma since mutations in *GNAQ* and *GNA11* are less common and genes involved in conjunctival melanoma such as *BRAF* have been described. The genetic spectrum of conjunctival melanoma, however, includes frequent mutations in the *BRAF*, *NRAS* and *TERT* promoter genes, which are found in cutaneous melanoma as well. The *BRAF* status of the tumor is not correlated to prognosis, whereas the *TERT* promoter gene mutations are. Clinical presentation, histopathological characteristics and copy number alterations are associated with survival in ocular melanoma. Tissue material is needed to classify ocular melanoma in the different subgroups, which creates a need for the use of non-invasive techniques to prognosticate patients who underwent eye preserving treatment.

INTRODUCTION

The first known description of uveal melanoma (UM), a specific form of ocular melanoma, dates from 1868, described by the German ophthalmologist and otolaryngologist Hermann Knapp.[1] Various subtypes based on cell type and pigmentation among other characteristics were later described in 1882 by Austrian ophthalmologist Ernst Fuchs. He also stated that enucleation was the treatment of choice, a treatment that is still used currently.[2] UM was a rare disease in that century; it still is, but the incidence is rising.[3-5]

Currently, ocular melanoma is the second most common type of melanoma after cutaneous melanoma and comprises 3–4% of all melanomas in the United States followed by mucosal melanoma.[3,4] Ocular melanoma can be divided into uveal and nonuveal ocular melanoma. Uveal melanoma (UM) is the largest group of ocular melanoma and consist of choroid, ciliary body, and iris melanoma. Nonuveal melanomas are all conjunctival melanomas (CM).[3] In almost all cases, one eye is affected because bilateral ocular melanoma is only reported in 0.1%.[4,5] Mean age of diagnosis in UM and CM is comparable (61.4 and 61.7 years old, respectively) in Caucasians,[4] although the age of onset of UM is lower in Asian patients.[6] The overall cancer-specific relative survival in UM is slightly higher compared to CM whereas the mean cancer-specific survival at 5 years is equal.[4]

Uveal Melanoma

UM is the most common primary intraocular malignancy in adults in the Western world with an incidence of 5–7:1,000,000 people.[4,5] UM arises predominantly in the choroid followed by ciliary body and iris (Figure 1).[7,8] The prognosis of iris melanoma is favorable compared to melanoma of the choroid and ciliary body.[9,10] The 5-year overall survival of choroid and ciliary melanoma is 77–80% with a cancer specific 5-year survival rate of 76%. More than half of all patients develop metastases with a median survival of six months when metastatic disease is present in UM, whereas the melanoma related death in iris melanoma is 3–4%.[4,11-13] One study even showed a 15-year melanoma specific survival up to 100%.[10] Although both groups have resemblances in their genetic makeup, the involvement of certain genes is different as well as their clinical behaviour. Therefore, iris melanomas are considered a distinct subgroup within UM. UM used in the literature generally refers to posterior (choroid and ciliary body) UM.

UM can appear as (partly) amelanotic lesions as shown in Figure 2.

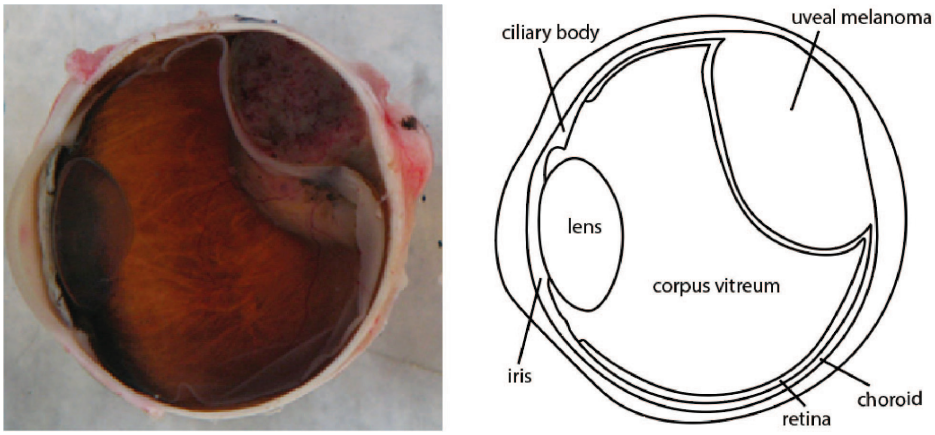

Figure 1. Section of an eye with a uveal melanoma in the choroid (**left**), schematic overview of the anatomy (**right**).

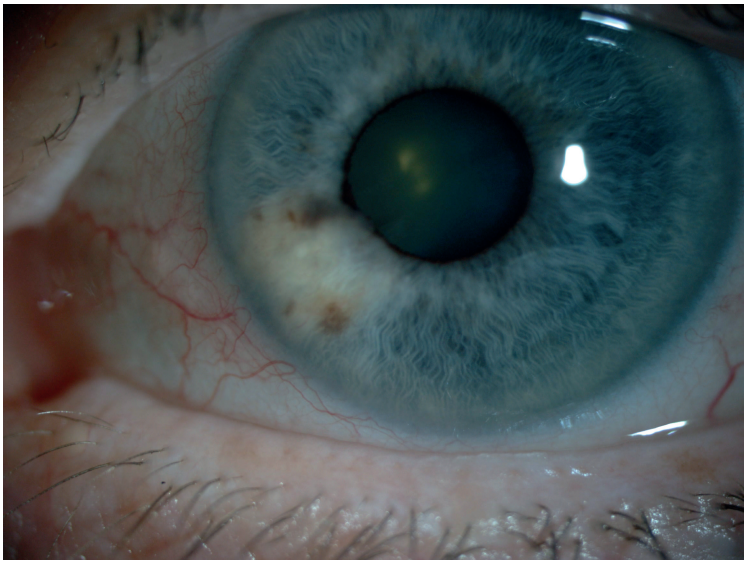

Figure 2. Partly amelanotic iris melanoma.

Treatment of primary UM consist of surgery, radiotherapy or a combination of these therapies.[4] A large randomized trial has shown that treatment modality has no effect on long-term survival.[14] However, in small UM, radiotherapy might have a beneficial effect over surgery regarding overall survival, although high-risk patients were not identified within this study.[15] Unfortunately, there is no adequate treatment available for meta-static UM. First line treatment with immunotherapy, using an anti-PD-1 monoclonal antibody named pembrolizumab, showed only positive results in a minority of patients.[16] Since *GNAQ* and *GNA11* genes, in which mutations occur in UM,[17] are related to the

MAPK-Erk Pathway, inhibitors of this pathway (MEK inhibitors) could have an effect of metastatic disease. However, clinical trials using MEK inhibitors in metastatic UM show contradictory results.[18,19]

Mutations in certain genes as well as chromosomal aberrations correlate with patient prognosis.[20,21] Not only are genetic and cytogenetic characteristics correlated to prognosis, but several clinical and histopathological characteristics are also associated with a higher risk of metastatic disease. Clinical and histopathological parameters correlated with a poor prognosis are larger tumor diameter, ciliary body involvement, mixed or epithelioid cell type, extracellular matrix patterns and high mitotic or Ki-67 proliferation index.[22]

Pediatric Uveal Melanoma

UM in children and young adults are described in less than 1% of all UM.[8,23-25] Like in adult UM, the tumor is most commonly primarily located in the choroid. Contrary to the frequency in adults, however, the frequency of iris melanoma is higher than melanoma originating from the ciliary body. A large cohort of 8033 UM patients described by Shields et al. showed in the age group of patients of 20 years or younger that 21% of developed UM are iris melanoma, whereas in adults, only 2–4% of UM consists of iris melanoma.[8] Other studies describe iris melanoma in about 20–25% of all UM patients in children and young adults.[24,26] It seems that females are at more risk to develop UM before the age of 25, although this was not statistically significant in cohort studies, probably due to the small size of the groups.[26-28]

Treatment of paediatric UM does not differ from the treatment in adults and includes enucleation, radiotherapy, resection and proton beam therapy.[24,25,27] Few children are treated with laser photocoagulation, transpupillary thermotherapy, gamma knife and photodynamic therapy.[26,27]

The prognosis of UM in children and young adults differs from adult patients. Children with UM have a favorable prognosis compared to young adults with UM (18 to 24 of age) with a 10-year survival rate of 92% and 80%, respectively.[27] With a 10-year survival rate of 93%, juvenile UM patients have a better prognosis compared to adults (65%).[24] Metastasis of UM (posterior and iris melanoma) in patients with age at diagnosis <21 years are described in 8–44%[8,24,25,29,30] with congenital melanocytosis as a predictor of poor prognosis.[27] Patients with extraocular extension also have a significant higher risk of UM-related death, whereas ciliary body involvement or cell type had no effect on prognosis.[27] Although not statistically proven, it seems that females tended to have a worse survival compared to males.[27,28]

Conjunctival Melanoma

Conjunctival melanoma (CM) arises from melanocytes in the epithelium of the conjunctival membrane and account for less than 10% of all ocular melanoma.[4,5,9] The incidence of CM is 1–2 per 1,000,000 people in the Western world with an increasing trend. [31,32] Malignant lesions account for 6–30% of all conjunctival lesions, with squamous cell carcinoma and melanoma being the most frequent.[31,33] CM most often arise from primary acquired melanosis (PAM) but CM originating from nevi or de novo also occurs (Figure 3).[34,35] Conjunctival melanoma in children is rare, a systematic review of the literature from 2019 by Balzer et al. described 32 patients with conjunctival melanoma with an age of onset before 18 years.[36]

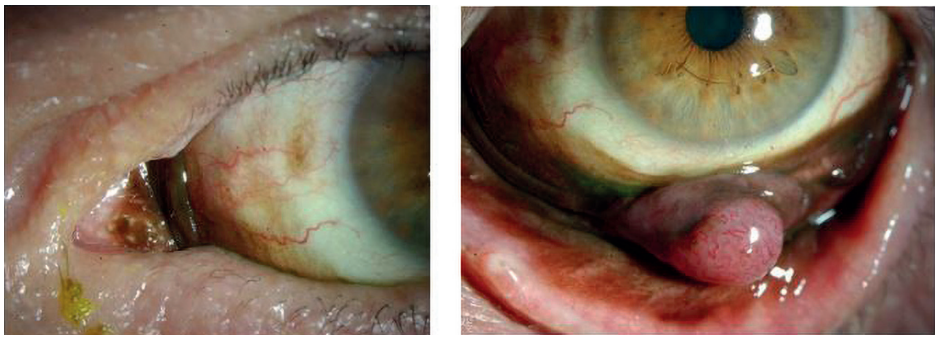

Figure 3. Primary acquired melanosis (**left**), conjunctival melanoma (**right**) in the same patient.

Treatment consist of excision in most cases, preferably in combined with cryotherapy.[4,33] Cryotherapy as single treatment is uncommon as well as enucleation Other less used treatments are topical or injection chemotherapy, exenteration, plaque radiotherapy, external beam radiotherapy and systemic chemotherapy.[33]

Risk factors for metastasis (nodal and distance) are a higher tumor thickness, histologic ulceration and the presence of mitotic figures.[37] 12–26% of patients develop metastasis within 5 years after diagnosis.[38,39] Distant metastases were found in the liver, lung, brain or elsewhere. Local recurrence occurs in almost one third of patients.[38] Like in uveal melanoma, no standardized therapy is available for disseminated disease. However, small case series show the potential use of target therapy and immunotherapy in advanced local and metastatic conjunctival melanoma.[40,41]

GENETICS OF OCULAR MELANOMA

Chromosomal aberrations are a key feature of genomic instability of cancer cells and the observation of chromosome 3 loss in metastasizing UM by Prescher et al. in 1996 was a cytogenetic hallmark for UM.[42] With the introduction of new high throughput DNA sequencing techniques, replacing traditional karyotyping, a number of genes mutated by the different types of ocular melanoma were discovered. Their role in initiation or progression of the disease was investigated and will be discussed in the next sections for some of these genes.

Uveal Melanoma

The most frequently mutated genes in UM are *GNAQ* and *GNA11*. Mutations in these genes occur in 71–93% of all UM tumors; we and others have shown that they have no predictive value.[22,43-45] Mutations occur most often in the 209 residue of exon 5 although mutations in amino acid 183 in exon 4 are also described.[45] *GNAQ* and *GNA11* are involved in the Gα signaling pathway and are mutually exclusive in the vast majority of tumors. Other frequently mutated genes in UM are *BAP1*, *SF3B1* and *EIF1AX*.[21,46]

Prognosis of UM patients can be predicted with the mutation status of secondary driver genes *EIF1AX*, *SF3B1* and *BAP1* which are almost always mutually exclusive. Patients with a *BAP1* mutation or absent BAP1 expression with immunohistochemistry (IHC) have a high metastatic risk while patients with an *EIF1AX* mutation have a low risk of metastatic disease.[20,21,47] Mutations in *BAP1* are less frequently found in iris melanoma and not correlated with survival.[44] When looked at the chromosomal profile of UM, there is a decreased disease-free survival in tumors with loss of chromosome 3 (monosomy 3) which is associated with *BAP1* mutations.[20,22,47] Gain of chromosome 8q is a poor predictor as well.[48] Other chromosomal aberrations found in UM include chromosome 1p loss and gain of chromosome 6.[20,22] These chromosomal aberrations can be detected using karyotypes, fluorescent in situ hybridization (FISH) and single nucleotide polymorphism (SNP) array analysis.

In patients harboring a disomy 3 UM, two genes are mainly mutated: *EIF1AX* and *SF3B1.* In eukaryotes, *EIF1AX*, encoding for the X-linked Eukaryotic Translation Initiation Factor 1A protein, stimulates and stabilizes the ribosome and is involved in start codon recognition.[49-52] In UM, mutations in *EIF1AX* primarily occur as heterozygous amino acid substitutions in exon 1 and 2, causing an in-frame mutation affecting the proteins N-terminus.[53,54] As EIF1AX acts as a regulator for translation initiation, mutations herein result in wrong selection of start sites, which might cause suppressed translation of canonical transcripts or upregulation of oncogenes.[53,55] However, the precise biological function and its contribution to tumorigenesis is not fully understood. Although the

heterozygous mutation is located on the X-chromosome, in females, the wild-type allele is silenced through selective X-chromosome inactivation, resulting in mutant transcripts only.[53]

Yavuzyigitoglu et al. have shown that patients harboring an *EIF1AX* tumor have a good prognosis, as these tumors harbor a low risk of metastasis.[23] *EIF1AX* mutations are reported to occur in 8% to 19%.[56]

SF3B1 encodes for a part of the spliceosome, splicing factor 3 subunit 1. The spliceosome is responsible for splicing noncoding introns from precursor mRNA at specific splice sites, leaving only the exonic sequence.[57] As part of the spliceosome, the SF3b complex recognizes branch point sites on precursor mRNA at which U2 snRNP (small nuclear ribonucleoprotein) is recruited. SF3b facilitates the interaction between the branch point site and U2 snRNP by protein crosslinking, after which the spliceosome is catalyzed.[58-60] The majority of recurrent hotspot mutations in SF3B1 occur at the edge of the C-terminal HEAT domains, near the precursor mRNA binding region and might be important for RNA or protein interactions.[57,61]

Mutations in *SF3B1* lead to aberrant transcripts [58], primarily caused by alternative 3' splice site selection upstream of the canonical splice site, coincided by misregulated branch point usage.[62] As a result, mutations in spliceosome components, such as *SF3B1* mutant tumors, can have alternative 3' acceptor splice sites, alternative cassette exons and intron retention in protein coding and noncoding genes as shown by Furney et al.[63]

Yavuzyigitoglu et al. reported UM patients harboring an *SF3B1* mutation were diagnosed younger at 54.5 years than patients harboring an *EIF1AX* or *BAP1* mutated tumor, diagnosed at 64 years.[21] They reported that these patients have an apparent risk of late onset metastases as 11 of 32 (34%) metastasized within 16 years (mean: 11.2 years after initial diagnosis).[23] Harbour et al. described that 18.6% of the UM mutations occur in *SF3B1*.[64]

Because of the small number of iris melanoma, the genetic background is not as extensively explored as posterior uveal melanoma. Although genes involved in posterior uveal melanoma are mutated in iris melanoma as well, there are some differences. Iris melanoma harbor *GNAQ*, *GNA11* and *EIF1AX* mutations while *BAP1* mutations and mutations in *SF3B1* are less common or rare.[43,44] A mutation in *BRAF*, a gene often mutated in cutaneous melanoma, was identified in iris melanoma.[44] Loss of chromosome 3 is described in iris melanoma as well as loss of 9p.[65] Moreover, aberrations of chromosome 1, 6 and 8, chromosomes that are involved in posterior uveal melanoma as well, were described in iris melanoma.[66,67]

More sequencing and larger UM patient cohorts identified less prevalent recurring genes. Mutations in *PLCB4*, a downstream effector of Gαq signaling are described in <10% of uveal melanoma.[68] A study aimed at identifying gene mutations in 139 UM showed mutations in *GNAQ* and *GNA11* in 93% being mutually exclusive except for one UM harboring a *GNAQ* and *GNA11* mutation. Mutations in *PLCB4* (2%) were found in tumors with or without a *GNA11* mutation, whereas mutations in *CYSLTR2* (5%) were identified in UM with no mutation in one of the other genes. Deletions in spliceosome factors *RBM10*, in-frame deletions of *SRSF2* and homozygous deletion *SF3A1* were found in only a few tumors[69]. Mutations in *SRSF2* were all heterozygous in-frame deletions and starting at residue 92 or 93, except for one case described in The Cancer Genome Atlas (TCGA) starting at 174.[70]

Conjunctival Melanoma

Mutations in *BRAF* are identified in 25%–35% of conjunctival melanoma of which the vast majority is the *BRAF* V600E mutation. This mutation can be identified using genetic testing, but immunohistochemistry is used as well.[34] The *BRAF* gene is involved in signal transduction and mutated in different types of cancer, most commonly in malignant melanoma. Amino acid valine (V) at residue 600 is mutated and replaced by a glutamic acid (E) in cutaneous melanoma.[71] *BRAF* mutations were more often identified in conjunctival melanoma with a bulbar localization[34]. Apparently, cutaneous melanoma and conjunctival melanoma have an overlap in their genetic background. Other genes involved in the development of conjunctival melanoma are *TERT* promoter, *NRAS* and *NF1* in which pathogenic mutations are described.[35,72,73] *GNAQ* and *GNA11* mutations are identified, but not the activating hotspot mutations that occur in UM.[35] Amplification of chromosome 6 is found in more than half of the conjunctival melanoma. Moreover, alterations in chromosome 9q, 11q, 6p, 17p and 19 have also been detected.[39] *TERT* promoter mutations have recently been identified to correlate to metastatic disease.[74]

INHERITANCE OF UVEAL MELANOMA

Mutations or variants in genetic information can be passed from one generation to the next (inheritance) and cause a specific phenotype or disease. This is only possible if the mutation is present in the gametes, which is in general a germline mutation (Figure 4). Somatic mutations occur during embryogenesis or throughout life and are not present in the gametes and therefore not heritable.

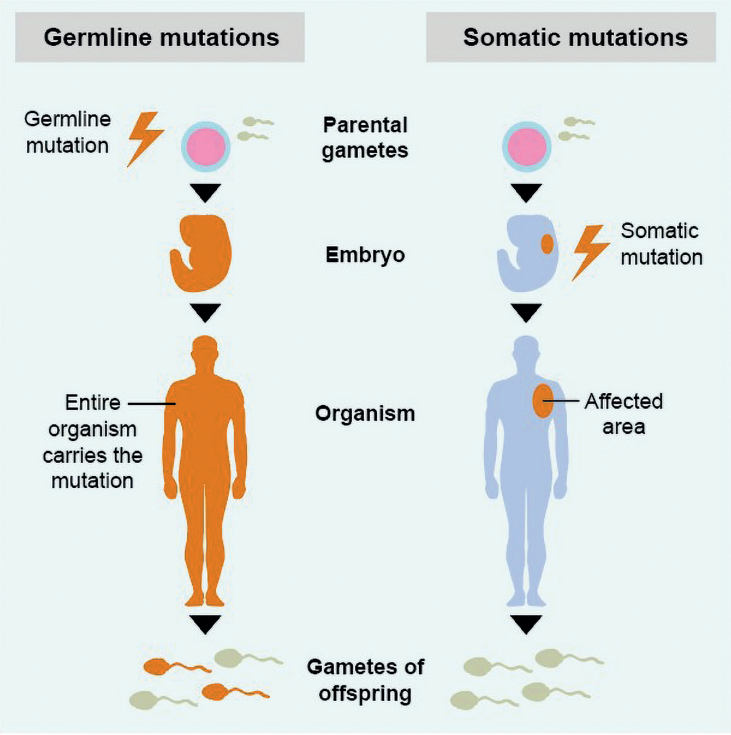

Figure 4. Schematic overview of germline and somatic mutations.

Only 2–4% of all uveal melanoma patients harbor a germline *BAP1* mutation[75-77] and although familial uveal melanoma is rare, *BAP1* has been identified as a predisposition gene for UM as well as a variety of other cancers.[78] When focused on familial UM, the incidence of *BAP1* germline mutations is higher and is reported up to 19%. Not only UM was described in these families but other cancers such as cutaneous melanoma and renal cell carcinoma were present in family members with this *BAP1* tumor predisposition syndrome (BAP1-TPDS).[79] Almost all UM in patients with a germline *BAP1* mutation have tumors that are located posteriorly, although one iris melanoma has been described.[80] In general, more cutaneous melanoma and ocular melanoma in the family history was reported in patients with uveal melanoma and a *BAP1* germline mutation compared to patients without this germline mutation. Moreover, germline mutated *BAP1* carriers have a larger tumor diameter and more frequently reported ciliary body involvement. Multivariate analysis did not show that germline *BAP1* mutations are an independent risk factor for the development of metastasis.[75] When metastasis-free survival of UM patients with a germline *BAP1* mutation was compared to those with a somatic *BAP1* mutation, it was shown that the germline *BAP1* mutated group has a more favorable prognosis.[81] In contrast, another study showed that germline *BAP1* mutations occur more often in metastatic ocular melanoma compared to non-metastatic ocular melanoma,

even though this difference was not significant and not adjusted for the greater risk of metastatic disease in *BAP1*-mutated UM in general.[82] The median age of diagnosis does not differ between patients with a somatic or germline *BAP1* mutation.[81]

The four main tumor types strongly associated with the *BAP1*-tumor predisposition syndrome (*BAP1*-TPDS) are uveal melanoma, mesothelioma, cutaneous melanoma and renal cell carcinoma.[83] The frequency of *BAP1* germline mutations is higher in families with cutaneous and uveal melanoma compared to families without uveal melanoma.[82] In families with a positive family history of UM, the frequency of *BAP1* germline mutations was 22%.[77] Accordingly, an accurate family history should be obtained when diagnosing new UM. In addition, it has been shown that germline null mutations in *BAP1* are more frequently observed compared to controls and the *BAP1*-TPDS is probably underreported.[84] Therefore, *BAP1* germline testing might be useful in case of familial UM or the occurrence of other cancers in a patient's family history. Other germline mutations described in UM are mutations in the TP53 gene, although these are rare and the role of these mutations should be elucidated.[85] TP53 mutations associated with UM and breast cancer in a family are already described in 1905. This was probably in the context of the Li–Fraumeni syndrome.[86]

PROGNOSIS

Uveal Melanoma

Several clinical and histopathological characteristics of UM are used to predict patients' prognosis. Initially, it was found that histopathologic features as cell type, largest tumor diameter and the location of anterior margin were correlated to different risk class of melanoma-related survival.[87] Other predictors for poor outcome were scleral extension, mixed/epithelioid cell type, Ki-67 proliferation index, inflammatory phenotype, high mitotic figures and deeper scleral extension and the presence of extracellular matrix patterns.[22,87,88] However, some of these characteristics are not independent of each other. For example, larger tumors are more commonly found in the anterior choroid or ciliary body and feature epithelioid cells.[89]

Not only can clinical and histopathological characteristics of the tumor be used to predict patients' prognoses, but patient characteristics are also important factors. Patients who develop UM before the age of 21 have a better prognosis compared to middle-aged adults (until age 60) or older patients.[8,30] It should, however, be mentioned that tumor thickness and diameter was not equally distributed between all age groups.[8] Besides age at diagnosis, there are studies

showing male patients are at higher risk for the development of metastases than female patients.[90,91] Moreover, metastatic disease developed earlier in men and the survival rate from diagnosis of metastatic disease was lower.[90] This difference in sex as a risk factor was not detected in Asian populations and the metastasis-free survival was higher compared to previous mentioned studies.[6] Uveal melanoma does occur in pregnancy, although the survival rates are similar to non-pregnant women.[92]

Later on, genetic factors such as chromosomal aberrations and genetic mutations were added to improve prediction of patients' survival. It was shown that patients with UM and loss of chromosome 3 (monosomy 3)[93-95] and gain of chromosome 8q in the tumor had a significant poor prognostic influence.[22,94,95] In addition, monosomy 3 was an independent risk factor for the development of metastasis, and thus poor prognosis, when corrected for tumor diameter and tumor site. Nevertheless, this study did not show a correlation of histological cell type, extrascleral extension and tumor thickness to prognosis.[42] One of the methods used in cytogenetics to detect chromosomal aberrations is FISH. This technique is used in UM to confirm the use of chromosome 3 and 8 and their relation to prognosis.[96] Further research validates the fact that patients with a UM showing monosomy 3 have a significantly lower disease-free survival. In addition, a relation between concurrent loss of 1p and 3 and the risk of metastasis was shown. UM that harbor both chromosomal aberrations is at an even higher risk of developing metastasis than UM with solely loss of chromosome 3. There was also a relation between cell type and the existence of chromosome 3 loss or 6p gain.[93] Loss of 1p and 8 are significant prognostic factors independently.[91] In order to display these chromosomal aberrations, SNP-array analysis can be used. SNP-array analysis is a technique which is frequently used in UM research (Figure 5). For example, greater tumor thickness or larger diameter correlates with partial or complete monosomy 3.[97] This implies that the histopathological risk factors previously described are not independent of the genetic background of the primary tumor. This study also showed that the patients with UM harboring partial monosomy have better prognoses compared to those with complete monosomy 3,[97] although later studies showed no significant difference in survival between patients with monosomy 3 or partial loss of chromosome 3 of the primary UM. In addition to these findings, loss of heterozygosity of chromosome 3 is even more important than monosomy 3 by itself.[98]

The role of BRCA1-associated protein (BAP1), located on chromosome 3, was proposed to have a role on the prognosis of UM about a decade ago. It was found that somatic mutations in *BAP1* were frequently present in metastasizing UM.[99] Immunohistochemistry (IHC) can be used to detect the presence of BAP1 protein expression (Figure 6). Nuclear BAP1 expression is strongly correlated

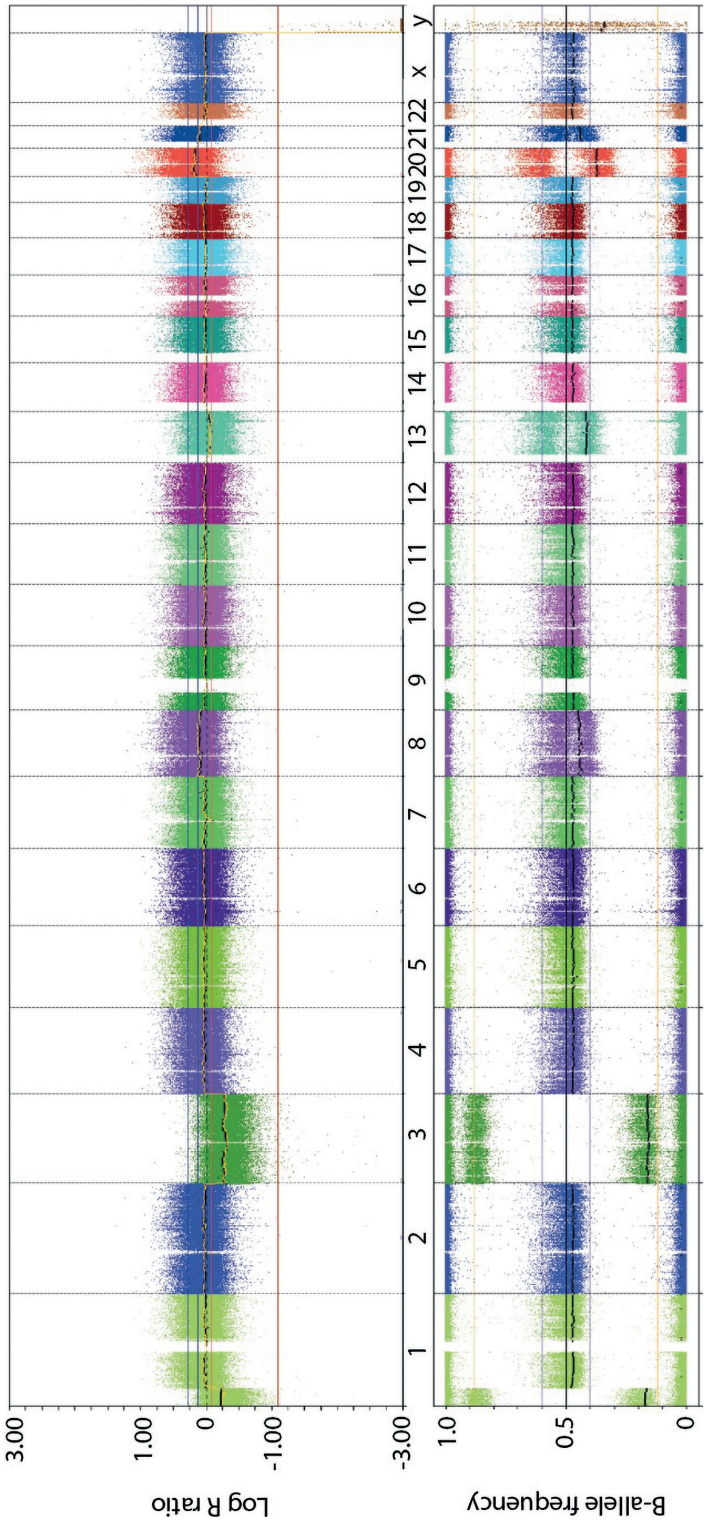

Figure 5. SNP-array profile of a uveal melanoma with partial loss of chromosome 1p and loss of chromosome 3. The *x*-axis represents the chromosomes. The upper figure displays the Log R ratio; in the lower figure, the B-allele frequency is showed.

with patient survival and metastatic rate; lack of expression is a risk factor for the development of metastasis and poor prognosis.[100,101] It was shown that *BAP1* mutations often results in the absence of BAP1 expression using IHC. Moreover, there is an association of BAP1 loss and monosomy 3 of the primary tumor.[102]

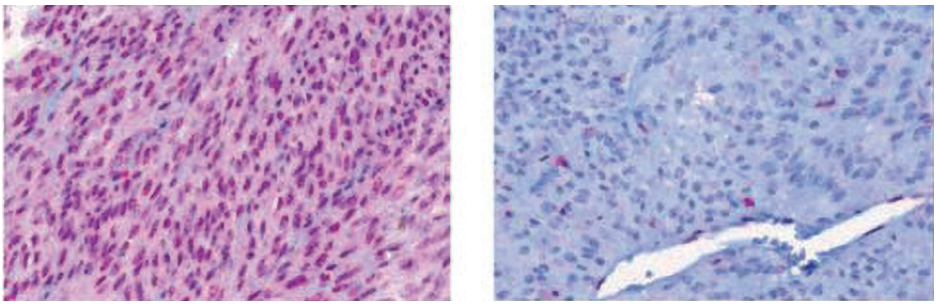

Figure 6. Microscopic overview of uveal melanoma with BAP1 expression 400x (**left**) and absence of BAP1 expression 400x (**right**).

When looking at specific gene mutations, there are several other genes described which can be used for prognostication besides *BAP1*. It has been shown that patients with UM harboring an *EIF1AX* mutation have prolonged survival and low risk of metastasis.[21,6] These somatic mutations mainly occur in UM with disomy 3.[21,46,55] Another mutation frequently found in disomy 3 UM is a hotspot missense mutation in *SF3B1* at codon 625.[21,55] Mutations in this gene, encoding subunit 1 of splicing factor 3b, are almost in all cases affecting codon 625, but mutations in K666 or K700 are also described.[53,54,63,108] Mutations in *SF3B1* are associated with alternative splicing of a wide range of target genes.[63] These findings were also identified in RNA sequencing data.

The clinical relevance of these splicing events is not completely clear, but it has been shown that *SF3B1* mutated tumors are at risk to metastasize. Patients with UM harboring an *SF3B1* mutation can develop late onset metastases. Metastases develop in most patients after 5 years, and metastatic disease can occur even after 10 years. This is in contrast with BAP1-mutated UM, in which metastases are mainly diagnosed within 5 years after diagnosis.[21]

Not only have chromosomal aberrations and mutation status of the tumor have been used to classify uveal melanoma patients, but a classification can also be performed with gene expression profiling. Two profiles can be distinguished, with class 1 being tumors with a good overall survival and low metastatic risk, whereas class 2 tumors are more likely to metastasize.[103] The ability to differentiate two groups of UM based on gene expression profiling correlating with survival was also shown in other studies.[104,105] This subgroups classification is not only based on gene expression profiling but corresponds with mutational

status and micro-RNA expression as well. These different mi-RNA expression profiles are probably not caused by the copy number state of the primary tumor but act as an independent process.[106] This mi-RNA expression profile can contribute to the prediction of patient prognosis. When looked to overall survival, the upregulation or downregulation of certain mi-RNAs have a prognostic value in patients with UM.[107]

Iris Melanoma

The prognosis of iris melanoma is favorable compared to posterior uveal melanoma. A large cohort of more than 1000 iris melanoma showed that 3% of iris melanoma metastasized.[114] This finding is according to smaller cohort studies in which metastatic disease is present in 10% or less.[11,44,109,110]

The American Joint Cancer Committee (AJCC) on Cancer classification can be used to describe and predict patient outcome. Most patients (75%) are scored following the AJCC Classification eighth edition as T1 (limited to the iris), whereas tumor confluent with or extending into the ciliary body and/or choroid (T2) including scleral extension (T3) or extra scleral extension (T4) are less common.[11] The 10-year risk of metastatic disease has been shown to be 5% in T1 tumors. Iris melanomas that are classified as T4 showed a 33% estimate of metastasis at 5 years, although only 5% of all iris melanomas were T4 tumors in this study.[11] Extraocular extension and high intraocular pressure are described as risk factors for metastasis.[109] Histological cell type is a risk factor as well; mortality was lower in spindle cell melanoma compared to mixed and epithelioid cell melanoma.[108,110] Within different age groups, there is no significant difference in survival between children, middle-aged adults and older adults.[109] BAP1 status using immunohistochemistry was not found of predictive value.[44]

Recurrent disease was higher in patients treated with iodine-125 radioactive plaque therapy in which there was reduced cornea surface coverage by the plaque and the presence of glaucoma after treatment. These risk factors were not correlated with the metastatic rate.[111]

Regarding the good prognosis after treatment, it should be noted that overtreatment could be possible in patients with iris melanoma. A large cohort of suspicious melanocytic iris lesions showed the low potential for malignant transformation and good prognosis.[112] This indicates that an overestimation of favorable prognosis after treatment is possible in patients who could have underwent conservative treatment as well.

Conjunctival Melanoma

Local recurrence rates of conjunctival melanoma are described in 30%–58%.[40,120] Treatment with excision alone has a higher risk of recurrent disease[40,120] as

well as non-epibulbar location of the tumor.[120,121] The 5-year overall survival is 72% (melanoma-related survival 90%).[40] Metastasis are reported in literature in about a quarter of conjunctival melanoma patients.[39,113]

Metastasis of conjunctival melanoma occur to regional lymph nodes, but distant metastases are described as well.[39,40] Distant metastases are found in patients following lymph node involvement, but are also described in patients without lymph node metastasis.[40] A correlation between tumor thickness (>2 mm), ulceration and mitotic figure count (>1/ mm^2) and regional lymph node metastasis was found.[37,74,115] Tumor diameter was also correlated with the risk of regional metastasis in a Dutch cohort.[113] Cell type is an important risk factor since patients with mixed cell type tumors had a higher mortality compared to spindle cell CM.[114] When lymphangiogenesis is present, a higher recurrence and risk of metastatic disease is present.[114,116] However this might be a confounder since high lymphatic density was associated with risk factors that are described as independent factors previously such as greater tumor thickness and larger tumor diameter.[116] In patients who underwent sentinel lymph node biopsy, a positive biopsy was related with a higher incidence of distant metastasis and a worse disease specific survival,[37] and local recurrence is associated with a higher risk of melanoma-related death.[40] Similar findings were reported using a large Chinese cohort: a higher T stage using the AJCC staging system, greater tumor thickness, more quadrants involved, local resection and the absence of adjuvant therapy were associated with worse survival.[117]

Chromosomal status is also correlated with survival, and it has been shown that deletions on chromosomal 10q are correlated with metastatic disease.[39] *BRAF* mutations occur frequently in conjunctival melanoma, especially in the sun-exposed area of the bulbar conjunctiva. However, no association of survival and gene mutation status regarding *BRAF* and *KIT* was identified.[117] This was confirmed in another study in which no relation between *BRAF* mutation status and local recurrence, metastasis and death is observed.[34]

The presence of *BRAF* mutations might be important in the future because BRAF/MEK inhibitors could possibly play a role in treatment of metastatic disease, as they is used in cutaneous melanoma where *BRAF* mutations at the same residue are present.[118] *TERT* promotor mutations correlate with prognosis which could act as a therapeutic strategy in the future.[74] The survival in children appears favorable compared to adults.[36] However, the incidence in children and adolescents is low and the groups described in literature small.

NONINVASIVE TESTING

Tumor tissue is needed to predict patients' prognoses; prognostication of patients who undergo eye preserving treatment such as radiotherapy is not possible based on a genetic profile when no biopsy was taken. Biopsies of tumor tissue are invasive with an inherent risk. Therefore, there is a need for noninvasive tumor testing which can not only be used for diagnostic purposes, but also to monitor the disease with a biomarker in real time. For other cancer types, noninvasive testing is widely used for diagnostics and follow-up of patients and includes cell free DNA (cfDNA), circulating tumor DNA (ctDNA), circulating tumor cells (CTC), tumor-derived exosomes, tumor-educated platelets and micro-RNA. These so-called liquid biopsies can be withdrawn from plasma, urine and other body fluids. One of the advantages of liquid biopsies over tissue biopsy is that tumor heterogeneity is more represented and changes of the mutational landscape of the tumor over time could be detected.

In lung and breast cancer, cfDNA concentrations correlate with disease progression.[119,120] Moreover, tumor-specific mutations could be detected in cfDNA from plasma, indicating that this technique can be used as a diagnostic as well as predictive tool.[121,122] Methods to isolate CTCs are well investigated and it has been shown that the prevalence of CTCs in blood in patients of several metastatic cancer types was risen.[123]

The use of CTCs in UM seems to have a predicted value on overall survival, although only small studies have been performed. Patients with CTCs detected in early-stage uveal melanoma have a less favorable prognosis compared to patients in which CTCs were not detected.[124] In patients with metastatic UM, the CTC cell count and ctDNA levels were also correlated with progressive free survival. There was also a relation with clinical characteristics of the tumor and the level of CTCs and ctDNA. More CTCs and higher levels of ctDNA in the blood were detected when the tumor volume was higher. In case of miliary hepatic metastases, resembling many small diffuse metastases in the liver, the ctDNA and CTC count was higher. This correlation was not found in patients with extrahepatic metastases.[125] When looked at chromosomal aberrations in the primary tumor, the CTC and cfDNA cells show an overlapping genetic profile. Moreover, *GNAQ* and *GNA11* mutations were detected in ctDNA of UM patients. *CYSLTR2* and *PLCB4* mutations were detected in only two patients. The detection rate of ctDNA was much lower in patients with localized UM (27%, $n = 30$) compared to patients with metastatic disease (100%, $n = 7$).[126]

When looked at mRNA expression in the blood of patients with UM using reverse transcription PCR, it has been shown that the detection of CTCs with this method can be used for prognostication as well. mRNA expression of tyrosinase

and MelanA/MART1 were correlated with disease-specific survival and overall survival.[127] Moreover, tyrosinase expression is significantly different when the primary tumor was classified regarding the tumor size. Tyrosine expression was the highest in large tumors, and there was a direct correlation between CTC values and tyrosine levels. The overall survival and disease-free survival were also better in patients without tyrosinase expression in their blood.[128]

Another entity that can be used for non-invasive testing in patients with cancer are exosomes. Exosomes are nanosized extracellular vesicles containing proteins, RNA and DNA excreted by cells and have functional properties.[129] Although UM is a relatively small tumor, the concentration of circulating exosomes derived from plasma of patients with metastatic uveal melanoma is higher compared to healthy controls. These exosomes contain melan-A and melanoma-associated micro-RNAS which support the theory that these exosomes are of metastatic melanoma origin.[130] These findings emphasize the fact that there is an enrichment of exosomes derived from cancer cells.

These methods give promising results of new techniques that can be used in the prognostication of patients with UM in a noninvasive manner. However, challenges will be faced due to tumor size and the heterogeneity in affected genes, especially the non-hotspot mutations that occur in *BAP1*.

CONCLUSIONS

Although posterior uveal melanoma, iris melanoma and conjunctival melanoma are all ocular melanoma, they are distinct subtypes. The genetic profile of the type of ocular melanoma differs from one to the other. Mutations that are common in posterior UM such as *GNAQ* and *GNA11* are described in iris melanoma but in lower frequency.[44] Moreover, mutations in *BRAF* were detected in only one iris melanoma,[44] whereas *BRAF* mutations are common in conjunctival melanoma.[34] Germline mutations in *BAP1* are described in UM, but conjunctival melanoma is not part of the *BAP1* tumor predisposition syndrome. In familial UM as well as families in which UM is present and family members known with malignant mesothelioma, cutaneous melanoma, clear cell renal cell carcinoma and basal cell carcinoma, an underlying germline mutation in *BAP1*, can be present. Therefore, it is recommended to test for germline *BAP1* mutations when the family history is suspect.

Not only the genetic background of these melanomas is different. The overall survival of patients with ocular melanoma of the different subtypes differs broadly. The prognosis of posterior UM is poor compared to iris melanoma and the metastatic site differs between UM and conjunctival melanoma. In

conjunctival melanoma, metastases to regional lymph nodes are described frequently, whereas UM primarily metastasize to the liver. The underlying pathogenesis of difference in survival is not yet clarified. It has been shown that *BAP1* mutations are correlated with poor prognosis in UM patients.[100] Since about half of all patients with posterior UM harbor a mutation in the *BAP1* gene, prognosis is poor. *SF3B1* mutations, correlated with late onset metastases, have been found more often in posterior UM compared to iris melanoma.[21,44] Based on the genetic profile, the difference in survival could be explained.

The time of diagnosis of iris melanoma is probably in an earlier stage compared to posterior UM since patients can detect changes in the iris. Posterior UM, in contrast, can be detected without any clinical symptoms by routine clinical examination. Loss of vision can occur when the melanoma is present in the macular region or when retinal detachment is present. It seems that UM with retinal detachment at presentation carries a higher risk of metastases. However, this risk can be attributed to the larger tumor diameter and other tumor characteristics.[131] Retinal detachment is therefore not a risk factor on its own. It is possible that these tumors are more aggressive, and therefore are detected at a larger tumor size. However, the time of diagnosis is probably earlier compared to tumors that do not give rise to any clinical symptoms. Therefore, it cannot be stated that iris melanoma has a better prognosis compared to posterior UM due to a probably earlier time of diagnosis.

Conjunctival melanoma and UM are both rare in children; however, the limited data suggest that survival in children seems better in both groups. Unfortunately, no current treatment is available for metastatic disease of ocular melanoma. In UM, liver resection is possible in only a few cases, but no validated systemic treatment is currently used. Targeted treatment for conjunctival melanoma harboring a *BRAF* mutation could be considered since the genetic profile is similar to that of cutaneous melanoma in which BRAF/MEK inhibitors are used. Further genetic and molecular testing is needed to gain more insight in ocular melanoma and hopefully lead to targets for therapeutic use.

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Chapter 1.2

Scope of this thesis

In this thesis the different types of ocular melanoma and their genetic backgrounds are described. The heredity of uveal melanoma (UM) is discussed and the possible use of targeted next-generation sequencing is shown.

An overview of the different types of ocular melanoma is given in **chapter 1**. Ocular melanoma can be divided in uveal melanoma and non-uveal melanoma such as conjunctival melanoma. The clinical characteristics of each entity are described as well as the different underlying genetic alterations. It has been shown that somatic mutations that occur in the primary tumours correlate with prognosis. In some patients a germline mutation in the *BAP1* gene underlies the development of UM. This *BAP1* tumour predisposition syndrome (*BAP1*-TPDS) is not only associated with UM but also other malignancies can develop in family members harbouring this germline *BAP1* mutation.

Primary tumour tissue is needed to evaluate the genetic risk profile. Since eye persevering treatment is common there is a need to develop alternative methods to detect tumour specific genetic changes using a non-invasive method. The use of cell free DNA and exosomes could play a role to determine genetic alterations in UM patients.

In **chapter 2.1** the gene mutations in iris melanoma and iris nevi are studied. The genetic profile of posterior UM and iris melanoma have similarities although there are other genes involved in iris melanoma such as *NRAS*, *BRAF*, *PTEN*, *c-KIT* and *TP53*. No correlation between survival and BAP1 status of the tumour was found whereas *BAP1* mutations in posterior UM are associated with poor prognosis. **Chapter 2.2** focusses on conjunctival melanoma and their specific genetic profile. Mutations in the *BRAF* gene, involved in cutaneous melanoma as well, are common and exposure to ultraviolet light leads to specific genetic mutations in *TERT*. Moreover a correlation between *TERT* promotor mutations and metastatic disease was observed. **In chapter 2.3** a phenomenon called chromothripsis, in which parts of chromosomes are re-arranged, is described in UM. Chromothripsis was detected in seven UM patients using SNP-array analysis. In one patient there was chromothripsis of two chromosomes. No statistical analysis regarding tumour characteristics and genetic mutations was performed due to the small number of cases. **Chapter 2.4** continues with a gene that is involved in splicing of DNA. This *SRSF2* gene is mutated in other cancers as well and the role of this gene in UM is described in this chapter. Patients were selected based on the chromosomal profile of the primary tumour since the chromosomal aberrations occurring in UM are correlated with genetic mutations in *SF3B1*, *BAP1* and *EIF1AX*. Patients were selected based on a genetic profile correlating with *SF3B1* mutations but a wildtype status of this gene. This specific profile was of interest since *SF3B1* and *SRSF2* are genes that are both involved in splicing.

Chapter 3 shed a light on UM in children and young adults. The role of germline mutations in *BAP1* in this specific group is discussed. This study tries to elucidate the underlying genetic predisposition in children and young adults who develop UM. No high incidence of *BAP1* germline mutations was found in this study suggesting that other factors are involved in the development of UM at a young age. Also the clinical characteristics of these young patients were studied and it was shown that the prognosis of boys is favourable compared to girls as described previously.

In **chapter 4** the use of formalin fixed paraffin embedded (FFPE) tissue in the detection of copy number variation and mutation analysis is described. This material is often unsuitable for mutation analysis but the use of a targeted next-generation sequencing panel analysis is shown. The UM related genes are included in this study as well as SNP's on chromosomes of interest to evaluate copy number variations. This is a reliable technique to perform genetic analysis on both fresh tumour material as well as FFPE.

Chapter 5 contains the general discussion of this thesis. The differences and similarities between posterior UM, irismelanoma and conjunctival melanoma on a genetic level are explained. Not only somatic mutations in ocular melanoma are described, but the role of germline *BAP1* mutations is discussed as well. To evaluate the genetic profile of ocular melanoma primary tumour tissue is needed but recent developments in the use of non-invasive testing are promising. Cell-free DNA and exosomes contain features of their originating cells and could be used to elucidate the genetic background of primary ocular melanoma in a non-invasive matter.

Chapter 2

Genetics of ocular melanoma

Chapter 2.1

Genetic background of iris melanomas and iris melanocytic tumors of uncertain malignant potential

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ABSTRACT

Purpose
Uveal melanoma (UM) is the most common primary intraocular malignancy in adults. Iris melanoma comprises 4% to 10% of all UMs and has a lower mortality rate. The genetic changes in iris melanoma are not as well characterized as ciliary body or choroidal melanoma. The aim of this study was to gain more insight into the genetic background of iris melanoma and iris nevi.

Design
Multicenter, retrospective case series.

Participants
Patients diagnosed with iris melanoma or iris nevi who underwent surgical intervention as primary or secondary treatment. Methods: Next-generation sequencing of *GNAQ*, *GNA11*, *EIF1AX*, *SF3B1*, *BAP1*, *NRAS*, *BRAF*, *PTEN*, *c-Kit*, *TP53*, and *TERT* was performed on 30 iris melanomas and 7 iris nevi. Copy number status was detected using single nucleotide polymorphisms (SNPs) included in the next-generation sequencing (NGS) panel, SNP array, or fluorescent in situ hybridization. BAP1 immunohistochemistry was performed on all samples.

Main Outcome Measures
Mutation and copy number status were analyzed. Results of BAP1 immunohistochemistry were used for survival analysis.

Results
In 26 of the 30 iris melanoma and all iris nevi, at least 1 mutation was identified. Multiple mutations were detected in 23 iris melanoma and 5 nevi, as well as mutations in *GNAQ* and *GNA11*. Furthermore, 13 of 30 *BAP1*, 5 of 30 *EIF1AX*, and 2 of 30 *SF3B1* mutations were identified in iris melanoma. No correlation between *BAP1* status and disease-free survival was found. The iris nevi showed 1 *EIF1AX* and 3 *BAP1* mutations. Two of the nevi, with a *BAP1* mutation, were histologically borderline malignant. Mutations in *NRAS*, *BRAF*, *PTEN*, *c-KIT*, and *TP53* were detected in 6 iris melanomas and 4 iris nevi.

Conclusions
Mutations that are often found in uveal and cutaneous melanoma were identified in this cohort of iris melanomas and iris nevi. Therefore, iris melanomas harbor a molecular profile comparable to both choroidal melanoma and cutaneous melanoma. These findings may offer adjuvant targeted therapies for iris melanoma. There was no prognostic significance of BAP1 expression as seen in choroidal melanoma. Consequently, iris melanoma is a distinct molecular subgroup of UM. Histologic borderline malignant iris nevi can harbor

BAP1 mutations and may be designated iris melanocytic tumors of uncertain malignant potential

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults with an incidence of 7 in 1 000 000 people in the Western World.[1] Iris melanomas comprise 4% to 10% of all UM.[1-4] The observed and relative survival is higher compared with UM in general.[5] There is no difference in incidence between men and women, but UMs occur more often in the white population.[4,6] Treatment includes surgical resection, enucleation, brachytherapy, and proton beam irradiation.[7,8] Currently, no studies on targeted adjuvant therapies in primary or metastatic iris melanoma exist. The choice of treatment depends on tumor size, localization, and patient preference. Diffuse iris melanomas are difficult to recognize, causing a delay in diagnosis. Moreover, they have a greater risk of metastasis than nodular iris melanoma.[9,10] Other clinical risk factors for metastasis include elevated intraocular pressure, iris root or angle involvement, increased tumor thickness, older patient age, and extraocular tumor extension. The metastatic rate of iris melanoma is quoted as 1% to 10% at 5 years, 2% to 10% at 10 years, and 10% at 20 years of follow-up.[6,10] A metastatic rate of 11% at 5 years was described in a series of biopsied iris melanoma.[11] However, gene expression profiling of iris melanoma showed that 67% of iris melanoma exhibit a class I (low metastatic risk) gene expression profile and 33% exhibit a class II profile (high metastatic risk).[12]

Chromosomal abnormalities of iris melanoma are poorly characterized. Partial and complete loss of chromosome 3 were found in 41% to 45% and 15% to 29%, respectively.[7,13,14] Monosomy 3 was correlated with increasing patients' age.[13] Although chromosome 3 loss is described in UM as a risk factor for metastatic disease,[15] in iris melanoma this was only associated with a progressive disease in a univariate analysis. Chromosome 9p loss was reported in 35%.[7] Furthermore, loss of 1p and 6q, and gain of 6p, 8, and 8q have been described.[7,14] Abnormalities of chromosomes 5 and 18 have been reported.[16] Mutations in genes encoding the guanine nucleotidebinding protein G subunit alpha q and 11 (*GNAQ* and *GNA11*) and the genes *BAP1*, *SF3B1*, and *EIF1AX* are typical for UM.[17,18] *GNAQ* mutations are more common in ciliary body and choroid UM compared with iris melanoma.[19] The aim of this study was to elucidate the genetic background of iris melanoma and iris nevi and to ascertain whether iris melanoma constitutes a distinct molecular group among UM. Next-generation sequencing (NGS) and immunohistochemistry were used to identify mutations in genes that are involved in both uveal and cutaneous melanoma.

METHODS

Inclusion

Tissue was collected from patients with iris melanoma or iris nevi from The Royal Hallamshire Hospital (Sheffield, UK) and the Rotterdam Ocular Melanoma

Study Group (ROMS) database. The ROMS is a collaboration between the Erasmus Medisch Centrum (Rotterdam, The Netherlands) and The Rotterdam Eye Hospital (Rotterdam, The Netherlands). Patients with an iris melanoma or suspect iris nevi who underwent biopsy or enucleation between 1992 and 2016 were included. The study conformed to the tenets of the Declaration of Helsinki and was approved by the respective local ethics committees. Informed consent was obtained before treatment. All samples were reviewed by 1 of 2 ophthalmic pathologists (H.S.M. and R.M.V.) to ensure that all tumors were primary iris lesions. Patient charts were reviewed to ascertain diagnosis as primary iris melanoma, clinical, and follow-up data.

Immunohistochemistry

Immunohistochemical staining was performed with a BAP1-antibody (clone sc-28383, 1:50 dilution, Santa Cruz Biotechnology, Dallas, TX) on 4-mm sections of formalin-fixed paraffin-embedded tissue (FFPE). An automated staining system (VENTANA BenchMark ULTRA, Ventana Medical Systems, Tucson, AZ) was used following the protocol as described previously.[20] Only nuclear expression was scored because nuclear expression is prognostic relevant in UM.[20,21] Loss of expression was defined as absent BAP1 expression in the nucleus. DNA Isolation DNA was extracted from fresh and FFPE tumor tissue. DNA isolation from fresh material was performed using the QIAmp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA extraction from FFPE tissue was performed using lysis buffer (Promega, Madison, WI) and 5% Chelex (Bio-Rad, Hercules, CA) following the protocol as described previously (Smit KN, Combined mutation and CNV detection by targeted NGS in UM, Modern Pathology, in press). Tumor tissue was confirmed with flanking hematoxylineeosin slides. DNA samples were stored at 20°C.

Targeted Next-Generation Sequencing

Targeted NGS was performed using the Ion Personal Genome Machine and the Torrent Server (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. A panel including amplicons covering *GNAQ*, *GNA11*, *BAP1*, *SF3B1*, and *EIF1AX* was used. Moreover, *NRAS*, *BRAF*, *PTEN*, *c-Kit*, *TP53*, and *TERT*, genes that harbor mutations in cutaneous melanoma, were included. On chromosome 1, 3, and 8, amplicons that cover highly polymorphic regions were used to identify allelic imbalances (Smit KN, van Poppelen NM, Vaarwater J, et al. Combined mutation and copy number variation detection by targeted next-generation sequencing in uveal melanoma. *Mod Pathol*. 2018 Jan 12. doi: 10.1038/modpathol.2017.187. [Epub ahead of print]).

Mutation Analysis

Results from Ion Torrent NGS were analyzed using Torrent Suite Software Version 4.4.3 (Thermo Fisher Scientific, Waltham, MA) and Integrative Genomics

Viewer Version 2.3.68 (97) (Broad Institute, Cambridge, MA). All data were manually analyzed using Integrative Genomics Viewer for the selected 10 genes by 2 individuals. Mutations that occurred in more than 20% of the reads and with a minimal read count of 50 reads were called. When there was a low DNA concentration or when 1 of the hotspot mutations was present in less than 20% of the total read count, mutations with a percentage between 10% and 20% were called. Intronic, noncoding regions and synonymous mutations were excluded. These results were compared with the mutations from the Variant Call Format files. Mutations were validated using Sanger sequencing following a standardized protocol for FFPE material if material was available.

Copy Number Variation

Allelic imbalances were detected using the highly polymorphic regions on chromosome 3. This data was used to estimate the copy number variation. Furthermore, Nexus Copy Number software (BioDiscovery Incorporated, El Segundo, CA) was used to display copy number variations. Additional single nucleotide polymorphism (SNP) array or fluorescence in situ hybridization (FISH) data were used when available. Single nucleotide polymorphism array and FISH results were obtained as described previously.[22,23] If there was loss of chromosome 3p, this was defined as loss of chromosome 3.

Statistical Analysis

For statistical analysis, IBM SPSS Statistics Version 21 (SPSS for Windows, International Business Machines Corporation, North Castle, NY) was used. Kaplan-Meier analysis with log rank test was used for survival analysis. A P value < 0.05 was considered significant.

RESULTS

Patient Characteristics

Iris melanomas

Between 1992 and 2016, from 31 patients who were treated for iris melanoma at Erasmus MC, The Rotterdam Eye Hospital and by the Ocular Oncology Service at the Royal Hallamshire Hospital, tissue material was available. From the Royal Hallamshire Hospital Sheffield, 20 patients were included and 11 patients from the Erasmus MC and The Rotterdam Eye Hospital. One patient who developed liver metastasis after 34.3 months was excluded because of low tumor DNA concentrations, which made genetic analysis unreliable. There were 17 men (57%) and 13 women (43%) with a mean age at diagnosis of 47.1 years (range, 16.7-70.4 years). Fourteen patients were treated with iridocyclectomy (47%). All 10 patients from Erasmus MC and The Rotterdam Eye Hospital and 1 patient from

the Royal Hallamshire Hospital underwent enucleation (37%). Three patients were treated with local iris resection (10%), 1 with iridectomy (3%) and 1 with proton beam therapy (3%). This latter patient was treated with cryotherapy for increased intraocular pressure 47.8 months after primary treatment, followed by enucleation because of a blind painful eye.

Two patients (7%) received additional treatment with ruthenium plaque and proton beam therapy because of incomplete excision of iris melanoma. One patient received additional treatment (stereotactic radiotherapy), although the resection was histologically complete. In 2 patients (7%), recurrent iris melanoma developed after 28.6 and 15.5 months after the primary treatment, necessitating proton beam therapy and enucleation, respectively. In 1 patient, 37.0 months after additional treatment, diffuse recurrent iris melanoma with increased intraocular pressure developed and the eye was enucleated.

Three patients (10%) underwent trabeculectomy because of glaucoma (5, 5, and 11 years) before the diagnosis of iris melanoma. Two patients were clinically diagnosed to have an iris nevus at the time of trabeculectomy. In the third patient, pigment was seen preoperative. Biopsy of the iris 4 years later revealed a borderline malignant nevus, and iris melanoma was diagnosed after 7 years. In this patient, metastatic disease developed 21.3 months after primary treatment of iris melanoma. The other 2 patients who underwent trabeculectomy did not develop metastatic disease. One patient was clinically diagnosed with a nevus and received a Baerveldt Glaucoma Implant because of glaucoma approximately 1.5 years before the diagnosis iris melanoma was made. Because of the iris melanoma diagnosis, the Baerveldt Glaucoma Implant was surgically closed and the eye was enucleated 3 weeks later. Table 1 shows an overview of patient characteristics.

Table 1. Clinical characteristics of patients and histopathological tumor features

Clinical or histopathological feature	Iris melanoma (n=30)	(Atypical) Iris nevi (n=7)
Gender		
Male	17 (57%)	2 (29%)
Female	13 (43%)	5 (71%)
Age at diagnosis (years), mean (range)	47.1 (16.7-70.4) (n=30)	58.5 (0.2-78.3) (n=7)
Primary treatment		
Enucleation	11 (37%)	1 (14%)
Iridocyclectomy	14 (47%)	0 (0%)
Iridectomy	1 (3%)	1 (14%)
Local iris resection	3 (10%)	0 (0%)

Table 1. Continued.

Clinical or histopathological feature	Iris melanoma (n=30)	(Atypical) Iris nevi (n=7)
Excision	0 (0%)	2 (29%)
Proton beam therapy	1 (3%)	0 (0%)
Biopsy	0 (0%)	3 (43%)
Disease free survival (months), mean (range)	114.5 (13.8-239.3) (n=29)	67.7 (35.8-120.1) (n=7)
Metastasis		
No	26 (87%)	7 (100%)
Yes	2 (7%)	0 (0%)
Unknown	2 (7%)	
Tumor diameter (mm), mean (range)	4.0 (2.0-6.0) (n=21)	2.2 (2.2-2.3) (n=2)
Tumor thickness (mm), mean (range)	2.0 (1.0-4.0) (n=22)	1.7 (1.1-2.2) (n=2)
Diffuse iris melanoma	8 (26.7%)	1 (14%)
Cell type		
Spindle cell	12 (40%)	7 (100%)
Epithelioid	7 (23%)	0 (0%)
Mixed	11 (37%)	0 (0%)
Involvement corpus ciliare		
No	22 (73%)	6 (86%)
Yes	8 (27%)	0 (0%)
Not stated	0 (0%)	1 (14%)
Involvement anterior chamber		
No	21 (70%)	5 (71%)
Yes	9 (30%)	1 (14%)
Not stated	0 (0%)	1 (14%)
Involvement trabecular system		
No	16 (53%)	4 (57%)
Yes	14 (47%)	2 (29%)
Not stated	0 (0%)	1 (14%)
Extraocular extension		
No	30 (100%)	7 (100%)
Yes	0 (0%)	0 (0%)

The mean disease-free survival was 114.5 months, with a range from 13.8 to 239.3 months. Metastasis in the liver developed in 2 patients (7%) after 21.3 and 31.9 months. KaplaneMeier analysis showed no significant difference in disease-free survival between patients with a BAP1-positive tumor compared with a BAP1- negative tumor (P= 0.470) (Fig 1).

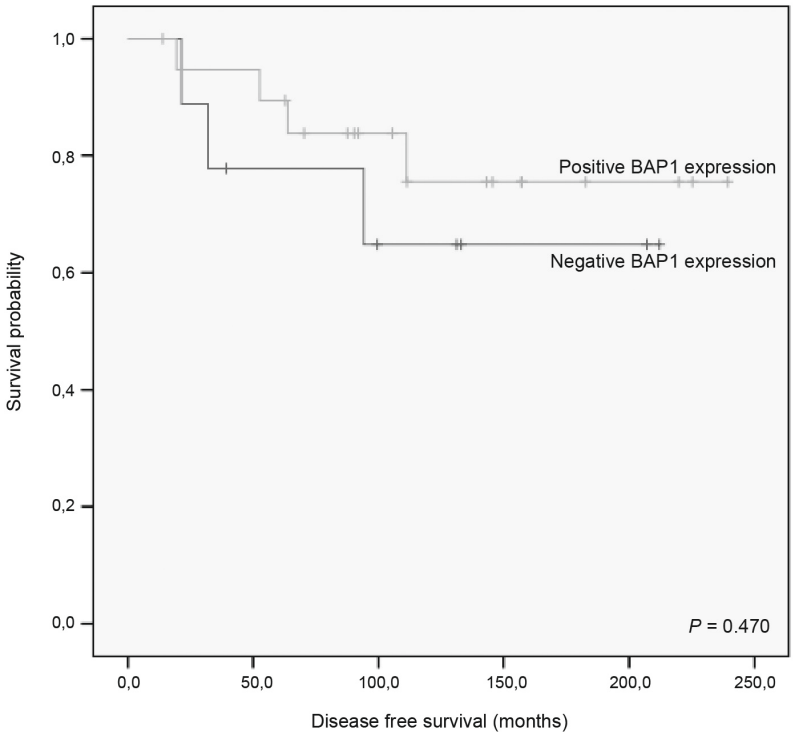

Figure 1. KaplanMeier curve showing disease-free survival for iris melanoma with a positive BAP1 expression compared with iris melanoma with a BAP1 negative expression. There is no significant difference between the 2 groups (P > 0.05).

Iris Nevi

The 7 patients with iris nevi from the ROMS database comprised 5 female patients (42%) and 2 male patients (29%) with a mean age at diagnosis of 58.5 years (range, 0.2-78.3 years). One patient underwent enucleation (14%), in 3 patients the nevi were excised in toto (43%), and 3 patients were biopsied (43%). None of these patients developed metastasis during follow-up (35.8-64.7 months). Six nevi were histologically classified as borderline malignant according to the Jakobiec and Silbert classification.[24]

Genetic Analysis

Ion Torrent data (Thermo Fisher Scientific, Waltham, MA) were analyzed for *GNAQ*, *GNA11*, *BAP1*, *SF3B1*, *EIF1AX*, *NRAS*, *BRAF*, *PTEN*, *C-KIT*, *TP53*, and *TERT*

promoter mutations. *TERT* promoter results were excluded for further analysis because of a read count T:p.Gln209Leu mutation (37%), 2 tumors harbored a c.626A>C:p.Gln209Pro mutation (7%), 1 tumor harbored a c.548G>A:pArg183Gln (3%), and 1 tumor harbored both a c.619G>A:pGly207Arg and a c.620G>A:p. Gly207Glu mutation (3%). *GNA11* was mutated in 9 iris melanomas (30%), which consisted of 6 c.626A>T:p.Gln209Leu (20%) and 3 c.547C>T:p.Arg183Cys mutations (10%). An *EIF1AX* mutation was identified in 5 tumors (17%): 3 c.5_6TT:p.Pro2Leu mutations (10%), 1 c.22G>A:p.Gly8Arg mutation (3%) and 1 c.44G>A:pGly15Asp mutation (3%). A c.1873C>T:p.Arg625Cys mutation in *SF3B1* was seen in 1 iris melanoma (3%), and a c.1858A>G:p.Met620Val mutation was seen in another tumor (3%). One or more *BAP1* mutations were found in 13 iris melanomas (43%).

For 3 iris melanomas, no mutation status of *NRAS*, *BRAF*, *PTEN*, *c-KIT*, and *TP53* was available. A *TP53* mutation was detected in 4 (13%), an *NRAS* mutation was detected in 3 (10%), a *PTEN* mutation was detected in 3 (10%), a *c-KIT* mutation was detected in 2 (7%), and a c.1781A>G:p.D594G *BRAF* mutation was detected in 1 iris melanoma (3%). The exact mutations are described in Table S1 (available at www.aaojournal.org). Four iris melanomas did not have a mutation in any of the tested genes. BAP1 immunohistochemistry was positive for all 4 of these samples.

In the iris nevi (n = 7), 4 *GNAQ* c.626A>T:p.Gln209Leu mutations (57%) and 1 *GNA11* c.626A>T:p.Gln209Leu (14%) were found. Three nevi, of which 2 borderline malignant, harbored 1 or more *BAP1* mutations (43%) and 1 had an *EIF1AX* c.16G>A:pGly6Ser mutation (14%). Mutations in *NRAS* were found in 4 nevi (57%), c-KIT was found in 3 nevi (43%), *PTEN* was found in 1 nevus (14%), and *TP53* was found in 1 nevus (14%). An overview of the mutations in iris melanoma and nevi are shown in Figure 2. Table S1 shows a detailed overview of the mutations that were detected.

Reliable Sanger sequencing results were obtained from 3 patients with a mutation in *PTEN*, *BRAF*, and *NRAS*. The mutations in *BRAF* and *PTEN* were confirmed. Surprisingly, besides the known *PTEN* mutation, another mutation in *PTEN* was detected with Sanger sequencing, a. c.703G>A:p.Glu235Lys mutation.

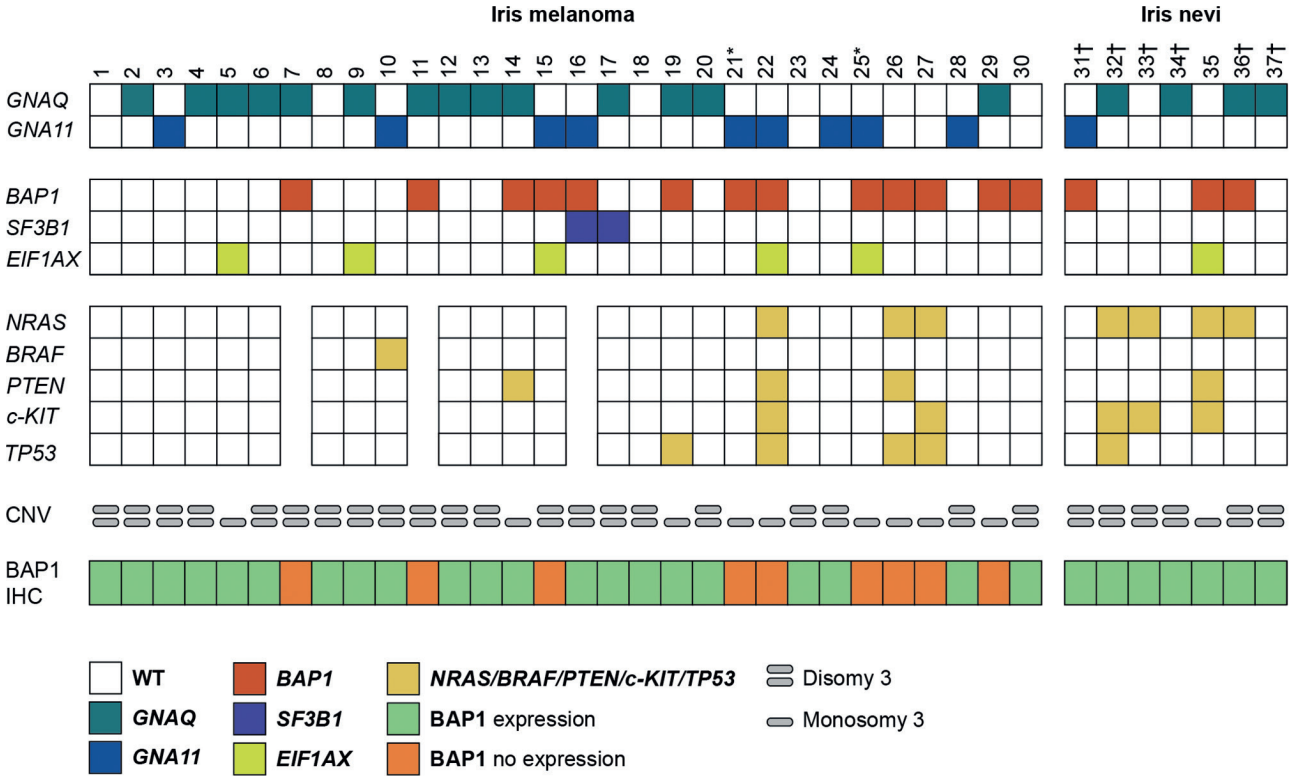

Figure 2. Overview of mutations, copy number variation, and BAP1 immunohistochemistry in all iris melanomas and nevi. The numbers represents all iris melanoma and nevus samples. In the first row, the known uveal melanoma (UM) hotspot mutations in GNAQ and GNA11 detected with next-generation sequencing (NGS) are displayed. The second and third row represent mutations that were identified with NGS in *GNAQ*, *GNA11*, *BAP1*, *SF3B1*, *EIF1AX*, *NRAS*, *BRAF*, *PTEN*, *c-KIT*, and *TP53*. The fourth row indicates the copy number variation of chromosome 3 detected with single nucleotide polymorphisms (SNPs) included in the NGS panel, fluorescent in situ hybridization (FISH), or SNP-array data. The fifth row represents BAP1 expression using immunohistochemistry. CNV = copy number variation; IHC = immunohistochemistry. *Metastasizing tumors. † Borderline malignant.

Immunohistochemistry

Immunohistochemical staining for BAP1 was performed on all iris melanoma and iris nevus sections. None of the iris nevi showed loss of BAP1 expression (Fig 3). BAP1 expression was positive in 21 iris melanoma samples (70%) and negative in 9 samples (30%). Six iris melanomas showed no BAP1 expression in >90% of the tumor cells, and in 2 cases loss of BAP1 expression was observed in 80% and 50% of the tumor cells, respectively. In the remaining BAP1 negative iris melanoma, part of the tumor (40%) consisted of epithelioid cells that lacked BAP1 expression and the spindle tumor cells showed BAP1 expression (Fig 4).

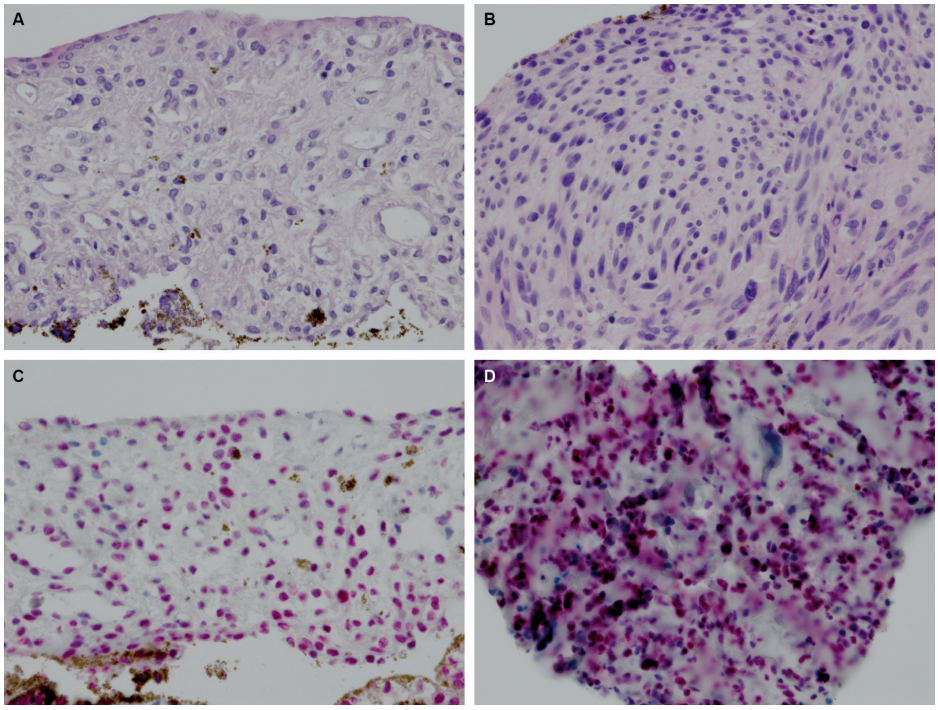

Figure 3. Histopathologic features of 2 iris nevi. A and C, B and D are the same nevus. Left nevus: monosomy 3, no BAP1 mutation was detected. Right nevus: disomy 3, a c.2146G>A mutation in BAP1 was identified. **A)** Hematoxylineeosin staining of an iris nevus (400x). **B)** Hematoxyline-eosin staining of an iris nevus (400x). This is an iris melanocytic tumor of uncertain malignant potential. **C)** BAP1 staining of an iris nevus, there is nuclear expression (400x). **D)** Positive nuclear BAP1 expression in an iris melanocytic tumor of uncertain malignant potential (400x).

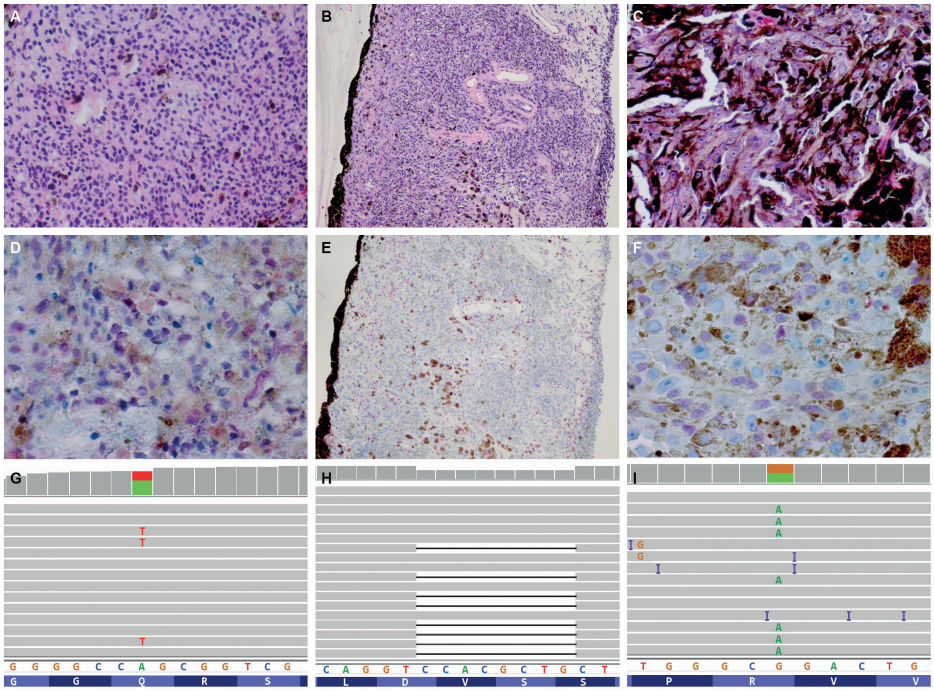

Figure 4. Histopathologic features and next-generation sequencing (NGS) results displayed in Integrative Genomics Viewer of 3 iris melanoma samples. **A)** Hematoxylineeosin staining of spindle tumor cells (200). **B)** Hematoxylineeosin staining of mixed spindle and epithelioid tumor cells (100). **C)** The tumor shows mixed spindle and epithelioid cells in a hematoxylineeosin staining (200). **D)** Positive nuclear BAP1 immunohistochemical expression in the tumor cells (400). **E)** Immunohistochemistry revealed no BAP1 expression (100) **F)** Positive BAP1 expression (immunohistochemistry) in spindle cells, absent BAP1 expression in epithelioid cells (400). **G)** The NGS results shows a c.548G>A:p.R183Q mutation in *GNAQ*. **H)** BAP1 c.312_319del:p.S104fs displayed in Integrative Genomics Viewer. **I)** Mutation in BAP1 c.1165C>T:p.R389.

Copy Number Status

Copy number loss of chromosome 3 was detected in 13 samples consisting of 12 iris melanoma and 1 borderline nevus. SNP-array data were available for 4 samples, and FISH was performed in 10 samples. The results from copy number detection using the SNPs from the NGS panel, SNP array, and FISH were consistent whenever more than 1 technique was available for analysis. The copy number status of cases 21 to 29 and 31 were evaluated by more than 1 technique. An overview of the copy number status, BAP1 immunohistochemistry, and BAP1 mutations is given in Figure 2.

DISCUSSION

To our knowledge, this is the largest study of genetic mutation analysis in iris melanoma and iris nevi for genes that are involved in uveal or cutaneous melanoma. Iris melanoma and nevi harbor mutations that are found in primary choroidal and cutaneous melanoma. In UM, prognosis is related to nuclear BAP1 expression,[20,21] whereas in this study, no significant association was found between nuclear BAP1 expression and disease-free survival in iris melanoma. Knowledge of the molecular profile is fundamental because potential therapies targeting the cutaneous melanoma signature could have clinical implications in iris melanoma.

Thirty iris melanomas and 7 iris nevi were analyzed for mutations in *GNAQ*, *GNA11*, *EIF1AX*, *SF3B1*, *BAP1*, *NRAS*, *BRAF*, *PTEN*, *c-KIT*, and *TP53* using NGS and BAP1 immunohistochemistry. In this cohort, more *GNAQ* mutations were detected compared with *GNA11* mutations, which is in line with previous reported mutations in iris melanoma.[25] A hotspot *GNAQ* or *GNA11* mutation was found in 23 iris melanomas (77%) and 5 iris nevi (72%). These mutations are the same hotspot mutations as described in UM. However, the mutation rate is lower compared with UM, in which a rate up to 93% is described.[18] Other genes that have been described in 3% to 7% of UM involving the Gas activating or Gai inhibitory adenylyl cyclase pathway, such as *CYSLTR2* and *PLCB4*,[26,27] could be involved in iris melanoma as well. It would be interesting to investigate whether *CYSLTR2* and *PLCB4* are mutated in iris melanoma with a *GNAQ* or *GNA11* wild-type profile, although no mutations in *CYSLTR2* have been found in an earlier study of 19 iris melanomas.[21] *GNAQ* and *GNA11* up-regulate the mitogen activated protein kinase pathway, as well as activating *BRAF* and *NRAS* mutations.[28] However, the mutation in *BRAF* (D594G) in our cohort did coexist with a *GNA11* mutation. Mutations in *BRAF* have been described in 9 of 19 iris melanomas, but these mutations were located at a different position than in our cohort.[29]

NRAS mutations were detected both with and without mutations in *GNAQ* and *GNA11*. Inhibition of MEK, a kinase in the mitogen-activated protein kinase, is an accepted treatment in specific metastatic cutaneous melanoma cases.[30,31] In contrast, response rates are lower in patients with metastatic UM.[31] Because iris melanomas harbor mutations in genes that are present in cutaneous melanoma, unlike UM, a study to elucidate the effect of MEK-inhibitors in this specific patient group may be warranted.

Mutations in *SF3B1* and *EIF1AX* were detected in 7% and 17% of cases, respectively. Considering the sample size, this is comparable to UM in which mutations in *SF3B1* vary between 10% and 24%, and *EIF1AX* mutated tumors are reported at approximately 20%.[32,33] A recent study of 19 iris melanomas showed

mutations in *EIF1AX*, but no mutations in *SF3B1*, *BRAF*, *NRAS*, and *c-KIT*.[25] However, mutations in *NRAS*, *BRAF*, *PTEN*, *c-KIT*, and *TP53* were found in both iris melanoma and nevi in our series. In The Cancer Genome Atlas, only 1 deletion in *c-KIT* has been described before. This supports our hypothesis that iris melanoma should be treated as a distinct subgroup of UM. An extra mutation in 50% of the alleles of *PTEN* was detected at confirmation testing with Sanger sequencing. Possibly, only 1 allele was covered with NGS, so that this mutation was not detected. In 4 iris melanomas, no mutations were detected, which supports our hypothesis of iris melanoma as a distinct subgroup. Other driver genes may be involved in the development of iris melanoma. These samples are subject for additional investigations.

Some studies suggest that mutations in uveal and iris melanoma might be associated with ultraviolet exposure.[25,34] However, in a whole-genome sequencing study of UM, no ultraviolet induced mutation signature was found.[35] In the current study, it is doubtful whether the mutations that we identified in *NRAS*, *BRAF*, *PTEN*, *c-KIT*, and *TP53* are related to ultraviolet light exposure because the primary tumors were located in different quadrants of the eye. Furthermore, the mutations that were found in the cutaneous melanoma associated genes were not predominantly C>T or CC>TT mutations, which are known to be caused by ultraviolet light damage.[36] Neither relations between the mutations and geographic differences nor regional effects were observed. Future studies are needed to validate the prevalence of mutations in *NRAS*, *BRAF*, *PTEN*, *c-KIT*, and *TP53* and their clinical relevance in iris melanoma.

It is known that chromosome 3 loss is correlated with *BAP1* mutations in UM.17 Therefore, copy number status was compared with *BAP1* mutations detected with NGS and BAP1 immunohistochemistry. Loss of chromosome 3 was detected in 13 samples, including 1 iris nevus. Chromosome 3 loss is described in iris melanoma, as well as abnormalities in chromosomes 1, 5, 6, 8, 9, and 18.[7,16] Loss of expression of BAP1 using immunohistochemistry is described in 43% to 50% of UMs[20,37] and in 1 of 3 iris melanomas.[25] In our study, immunohistochemistry for BAP1 was negative in 30% of iris melanomas, but a *BAP1* mutation was found in 43% using Ion Torrent next generation sequencing (Thermo Fisher Scientific, Waltham, MA). In 4 tumors with BAP1 expression, a mutation was detected with the sequencing results. Two of these iris melanomas had 2 copies of chromosome 3, which means that the wild-type allele can produce the BAP1 protein. For the other 2 cases with monosomy 3, it is possible that the mRNA is not degraded by nonsense-mediated mRNA decay. A non-functional BAP1 protein probably is expressed in these tumors. In all tumors with loss of BAP1 expression, mutations were detected with NGS.

In general, iris melanomas have a favorable prognosis compared with posterior UM.[5] BAP1 mutations and chromosome 3 loss are correlated with a poor prognosis in posterior UM.[15,20] Metastatic disease to the liver developed in 2 patients with iris melanoma (6.7%); 1 of them underwent trabeculectomy before the diagnosis. Both tumors harbored a *BAP1* mutation and had no BAP1 expression in the tumor cells. Nevertheless, this study demonstrates that there is no relation between BAP1 and prognostic outcome in iris melanoma (Fig 1). Therefore, the prognostic value of chromosome 3 and BAP1 status for iris melanoma is equivocal.

In the iris nevi, mutations in *GNAQ* and *GNA11* were identified. This is in line with the concept that mutations in these genes are an early event in tumorigenesis.[18] Moreover, a *GNAQ* mutation in an iris nevus has been described.[25] Mutations in *BAP1* were detected in 3 nevi, 2 of which were classified histologically as borderline malignant before knowing the *BAP1* status. One of these borderline malignant nevi was from an enucleated eye, and the other 2 were excised because they were also clinically suspect. Because these borderline malignant nevi were completely removed, it is uncertain if they would have developed into iris melanoma. Because most nevi showed borderline characteristics, the mutation status of typical nevi might be different. All borderline malignant iris nevi showed retained BAP1 expression. It is possible that the BAP1 expressing nevus cells obscured the small number of malignant subclones to confidently identify loss of BAP1 expression in these lesions. Further single cell analysis is warranted to resolve this issue. In case of a heterozygous mutation, the other allele can produce BAP1.

In conclusion, our study identified mutations in *GNAQ*, *GNA11*, *BAP1*, *SF3B1*, *EIF1AX*, *BRAF*, *PTEN*, *c-KIT*, and *TP53* in iris melanoma and iris nevi. These mutations were found in a cohort composed of samples from different institutes, with an even distribution. Borderline malignant iris nevi harbor mutations that confirm their clinical and histopathologic borderline malignant status. We think it would be better to designate such cases as iris melanocytic tumors of uncertain malignant potential, in line with the terminology used for uncertain cutaneous melanocytic lesions (e.g., melanocytic tumor of uncertain malignant potential).[38] This would be justified by a combination of histologic and molecular findings presented in this study. Because *BRAF*, *PTEN*, *c-KIT*, and *TP53* mutations are not typical for UM, iris melanoma and iris nevi should be considered a distinct subgroup not only on the basis of clinical and histopathologic criteria but also on the basis of molecular grounds.

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Supplementary Table 1. Overview of genetic mutations in iris melanoma and nevi

	GNAQ	GNA11	SF3B1	BAP1	EIF1AX	PTEN	NRAS	BRAF	KIT	TP53
1	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
2	c.626A>T: p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt	wt
3	wt	c.626A>T: p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt
4	c.626A>T: p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt	wt
5	c.626A>C: p.Q209P	wt	wt	wt	c.5_6TT: p.P2L	wt	wt	wt	wt	wt
6	c.626A>T: p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt	wt
7	c.626A>T: p.Q209L	wt	wt	c.1165C>T:p.R389C	wt	n.a.	n.a.	n.a.	n.a.	n.a.
8	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
9	c.626A>T: p.Q209L	wt	wt	wt	c.22G>A: p.G8R	wt	wt	wt	wt	wt
10	wt	c.626A>T: p.Q209L	wt	wt	wt	wt	wt	c.1781A>G: p.D594G	wt	wt
11	c.626A>C: p.Q209P	wt	wt	c.406G>T:p.E136*	wt	n.a.	n.a.	n.a.	n.a.	n.a.
12	c.626A>T: p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt	wt
13	c.626A>T: p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt	wt

Supplementary Table 1. Continued.

	GNAQ	GNA11	SF3B1	BAP1	EIF1AX	PTEN	NRAS	BRAF	KIT	TP53
14	c.626A>T:p.Q209L	wt	wt	c.1084G>A:p.D362N, c.1280G>A:p.G427E	wt	c.653G>A:p.C218Y, c.703G>A:p.E235K	wt	wt	wt	wt
15	wt	c.546_547TT:p.R183C	wt	c.373G>T:p.E125*	c.5_6TT:p.P2L	wt	wt	wt	wt	wt
16	c.619G>A:p.G207R, c.620G>A:p.G207E	c.626A>T:p.Q209L, c.638G>A:p.R213Q	c.1858A>G:p.M620V	c.1970G>A:p.R657K, c.61G>A:p.D21N, c.110G>A:p.S37N, c.454C>T:p.L152F	wt	n.a.	n.a.	n.a.	n.a.	n.a.
17	c.626A>T:p.Q209L	wt	c.1873C>T:p.R625C	wt	wt	wt	wt	wt	wt	wt
18	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
19	c.548G>A:p.R183Q	wt	wt	c.1963G>A:p.E655K, c.673G>A:p.D225N	wt	wt	wt	wt	wt	c.758C>T:p.T253I, c.899C>T:p.P300L
20	c.626A>T:p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt	wt
21	wt	c.626A>T:p.Q209L	wt	c.53T>G:p.L18R,	wt	wt	wt	wt	wt	wt

Supplementary Table 1. Continued.

	GNAQ	GNA11	SF3B1	BAP1	EIF1AX	PTEN	NRAS	BRAF	KIT	TP53
22	wt	c.626A>T:p.Q209L	wt	c.620G>A:p.R207Q, c.588G>A:p.W196*, c.76G>A:p.G26R, c.1258G>A:p.G420R	c.44G>A:p.G15D	c.124C>T:p.L42F, c.343G>A:p.D115N	c.52G>A:p.A18T	wt	c.2399T>C:p.L800P, c.1716C>G:p.D572E, c.1757G>A:p.R586K, c.2434G>A:p.G812S, c.1352C>T:p.S451F	c.289G>A:p.V97I
23	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
24	wt	c.626A>T:p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt
25	wt	c.546_547TT:p.R183C	wt	c.661G>T:p.E221*	c.5_6TT:p.P2L	wt	wt	wt	wt	wt
26	wt	wt	wt	c.1411G>A:p.A471T, c.1873G>A:p.E625K, c.2017_2019del:p.E673del, c.1060G>A:p.V354I, c.2183G>A:p.R728H	wt	c.385G>A:p.G129R	c.88G>A:p.D30N, c.196G>A:p.A66T	wt	wt	c.587G>A:p.R196Q, c.676G>A:p.G226S
27	wt	wt	wt	c.1430C>T:p.A477V, c.889G>A:p.E297K	wt	wt	c.226G>A:p.E76K	wt	c.1232C>T:p.T411I, c.1489G>A:p.V497M	c.46C>T:p.Q16*
28	wt	c.546_547A:p.R183C	wt	wt	wt	wt	wt	wt	wt	wt
29	c.626A>T:p.Q209L	wt	wt	c.312_319del:p.S104Rfs*19	wt	wt	wt	wt	wt	wt

Supplementary Table 1. Continued.

	GNAQ	GNA11	SF3B1	BAP1	EIF1AX	PTEN	NRAS	BRAF	KIT	TP53
30	wt	wt	wt	c.1000C>A:p.L334I	wt	wt	wt	wt	wt	wt
31	wt	c.626A>T:p.Q209L	wt	c.659+3_659+16del, c.91_93del:p.E31del	wt	wt	wt	wt	wt	wt
32	c.626A>T:p.Q209L	wt	wt	wt	wt	wt	c.413G>A:p.G138E	wt	c.1735G>A:p.D579N, c.1439C>T:p.S480F	c.91G>A:p.V31I, c.704A>G:p.N235S
33	wt	wt	wt	wt	wt	wt	c.388G>A:p.A130T	wt	c.1990G>A:p.G664R	wt
34	c.626A>T:p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt	wt
35	wt	wt	wt	c.466C>T:p.Q156*, c.670C>T:p.H224Y, c.2185C>T:p.Q729*, c.2039C>T:p.S680F, c.577C>T:p.H193Y, c.1930G>A:p.A644T, c.1243G>A:p.A415T, c.610G>A:p.D204N, c.1166G>A:p.R389H	c.16G>A:p.G6S	c.686C>T:p.S229L	c.407G>A:p.S136N	wt	c.1423C>T:p.Q475*	wt
36	c.626A>T:p.Q209L	wt	wt	c.2146G>A:p.D716N	wt	wt	c.427G>A:p.E143K	wt	wt	wt
37	c.626A>T:p.Q209L	wt	wt	wt	wt	wt	wt	wt	wt	wt

Mutations were identified in iris melanoma and nevi with next-generation and Sanger sequencing, build Human Genome version 19 was used. Number 1-30 are iris melanoma samples, 31-17 iris nevi. Abbreviations: wt = wildtype; n.a. = data not available.

Chapter 2.2

Molecular genetics of conjunctival melanoma and prognostic value of *TERT* promoter mutation analysis

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ABSTRACT

The aim of this study was exploration of the genetic background of conjunctival melanoma (CM) and correlation with recurrent and metastatic disease. Twenty-eight CM from the Rotterdam Ocular Melanoma Study group were collected and DNA was isolated from the formalin-fixed paraffin embedded tissue. Targeted next-generation sequencing was performed using a panel covering *GNAQ*, *GNA11*, *EIF1AX*, *BAP1*, *BRAF*, *NRAS*, *c-KIT*, *PTEN*, *SF3B1*, and *TERT* genes. Recurrences and metastasis were present in eight (29%) and nine (32%) CM cases, respectively. *TERT* promoter mutations were most common (54%), but *BRAF* (46%), *NRAS* (21%), *BAP1* (18%), *PTEN* (14%), *c-KIT* (7%), and *SF3B1* (4%) mutations were also observed. No mutations in *GNAQ*, *GNA11*, and *EIF1AX* were found. None of the mutations was significantly associated with recurrent disease. Presence of a *TERT* promoter mutation was associated with metastatic disease (p-value = 0.008). Based on our molecular findings, CM comprises a separate entity within melanoma, although there are overlapping molecular features with uveal melanoma, such as the presence of *BAP1* and *SF3B1* mutations. This warrants careful interpretation of molecular data, in the light of clinical findings. About three quarter of CM contain drug-targetable mutations, and *TERT* promoter mutations are correlated to metastatic disease in CM.

INTRODUCTION

Conjunctival melanoma (CM) comprises 5–10% of all ocular melanoma.[1-3] The majority derives from primary acquired melanosis with atypia (PAM), but infrequently, CM develops from a pre-existing nevus or de novo.[1,3-6] CM has an incidence of 0.2–0.8 per million[3,6,7] with an increasing trend.[3,8] The 5- and 10-years cumulative incidence of CM-related mortality is 17–31% and 22–59%, respectively.[5,7,9-11] The prognosis of ocular melanoma, including CM and uveal melanoma (UM), depends on clinical and histopathological features, as well as the molecular genetic make-up.[3,12,13] During the past decade, the molecular make-up of UM has been well-characterized, with UM harboring recurrent mutations in guanine-nucleotide-binding protein-Q (*GNAQ*), guanine-nucleotide-binding protein-alpha 11 (*GNA11*), BRCA-associated protein 1 (*BAP1*), splicing factor 3 subunit 1 (*SF3B1*), and eukaryotic translation initiation factor 1A (*EIF1AX*). *BAP1* and *SF3B1* mutations are associated with the development of metastasis in UM. After the diagnosis of metastatic disease, patients with UM have a survival between 2–9 months.[12] When CM has metastasized, there are also very limited treatment options.[1,13] Yet, although CM as well as UM are ocular melanoma, CM certainly do show overlapping features, including molecular abnormalities with cutaneous melanoma.[1,3,6,13,14] For example, in 25–40% of the CM driver v-raf murine sarcoma, viral oncogene homolog B1 (*BRAF*) V600E/K mutations are described.[1,2,6,13,15] This incidence is higher as compared to other mucosal melanoma, which harbor a *BRAF* mutation in only 12% of cases. Although a correlation between *BRAF* mutations and poor prognostic factors has been described in cutaneous melanoma, no predictive value is yet reported for mucosal melanoma.[16,17] Other genes in which mutations have been identified in CM are the neuroblastoma RAS viral oncogene homolog (*NRAS*), Kirsten RAS oncogene homolog (*KRAS*), neurofibromin 1 (*NF1*), telomerase reverse transcriptase (*TERT*), tyrosine protein kinase (*c-KIT*), *TP53*, and *BAP1*.[3,6,15,18] Mutations in *GNAQ*/*GNA11* have also been described, but these are not the known activating hotspot mutations at amino acid Q209 or R183, which occur in UM.[15,19] The genetic background of the melanoma originating from these different locations, emphasizes the differences between UM and CM, and the similarities between CM and cutaneous melanoma. Furthermore, in contrast to UM, some of the mutations frequently found in CM are amenable to targeted therapies. However, the prognostic value of these molecular abnormalities in CM is largely unclear. The aim of this study was to further elucidate the genetic background of CM within the spectrum of melanoma and to correlate these findings with the development of recurrences and metastasis.

RESULTS

Clinical and Histopathological Characteristics

Clinical and histopathological characteristics are listed in Table 1. Based on the availability of sufficient formalin-fixed paraffin-embedded (FFPE) tissue for DNA isolation, twenty-eight cases could be included. Gender was equally divided with 50% males and 50% females. The median age at the time of diagnosis was 64 years (range 16–89 years). Based on the clinical information, most tumors were (at least partly) located on the bulbar conjunctiva (16 cases, 57%) with involvement of the palpebral conjunctiva in 10 cases (36%), the fornix in 5 cases (18%), and the caruncle in 1 case (4%). The tumors had a median diameter of 0.7 cm (range 0.05–1.8 cm), with a median tumor thickness of 3.0 mm (range 0.18–7.70 mm). According to the Eighth Edition of the American Joint Committee on Cancer (AJCC) Cancer Staging,[20] twelve cases (43%) were pathological tumor (pT) stage pT1, including six pT1a cases (21%) and five pT1b cases (18%), and thirteen cases were pT2 cases (46%), comprising one pT2a case (4%), eleven pT2b cases (39%), and two cases (7%) with unknown tumor thickness. In three cases (11%), the pT status was unknown. In eighteen cases (64%), the melanoma were derived from PAM, four melanoma (14%) developed from a nevus, and three melanoma (11%) were de novo lesions. In three cases (11%), the origin could not be reliably determined, based on the pathology reports and the available clinical information.

Local recurrent disease occurred in eight patients (29%), between 6.8–156.8 months (median 29.3 months) after treatment. Nine patients (32%) developed metastatic disease between 1.7–49.2 months (median 14.3 months). Metastatic sites included lymph nodes (solitary or within the parotid gland) in all patients (n = 9), with metastatic disease in the orbit (n = 1), thyroid (n = 1), breast (n = 1), lung (n = 1), brain (n = 1), and spleen (n = 1). The thyroid and breast metastases were present in one patient, and the orbit and brain metastases were identified in one patient as well. The spleen and brain metastases were not histologically confirmed. The mean overall survival was 77.4 months (range 3.85–257.2 months), with a median of 62.8 months.

Table 1. Clinical and histopathological characteristics of the included conjunctival melanoma (CM)

Clinical characteristics	
Median age at diagnosis (years)	63 (16-89)
Gender	
Male	14 (50%)
Female	14 (50%)
Location	
Bulbar	16 (57%)
Palpebral	10 (36%)
Fornix	5 (18%)
Caruncle	1 (4%)
Metastasis	
No	19 (68%)
Yes	9 (32%)
Local recurrence	
No	20 (71%)
Yes	8 (29%)
Histopathological characteristics	
Median diameter (cm)	0.7 (0.05 -1.8)
Median tumor thickness (mm)	3.0 (0.18-7.70)
pT status	
pT1a	6 (21%)
pT1b	5 (18%)
pT2a	1 (4%)
pT2b	11 (39%)
pTx	5 (18%)
Origin	
PAM	18 (64%)
Nevus	4 (14%)
De novo	3 (11%)
Unknown	3 (11%)

Mutation Analysis

The specific mutations found per case are listed in Supplementary Table S1, with a summary of the mutations including correlation with metastatic and recurrent disease in Table 2. Fifteen CM cases (54%) showed a *TERT* promoter mutation. A mutation in the *BRAF* gene was identified in thirteen CM (46%), mostly affecting amino acid V600. *NRAS* mutations were seen in six cases (21%) and mutations in *BAP1* were identified in five CM (18%). A *PTEN* mutation was found in four CM (14%), and in two CM (7%), a mutation in *c-KIT* was identified. Interestingly, a p.Arg625His mutation in *SF3B1* was detected in one CM (4%). The diagnosis was unequivocally a CM in terms of both clinical and pathological reports. It was located in the nasal superior in the bulbar conjunctiva (Figure 1). None of the CM cases carried a mutation of *GNAQ*, *GNA11*, or *EIF1AX*.

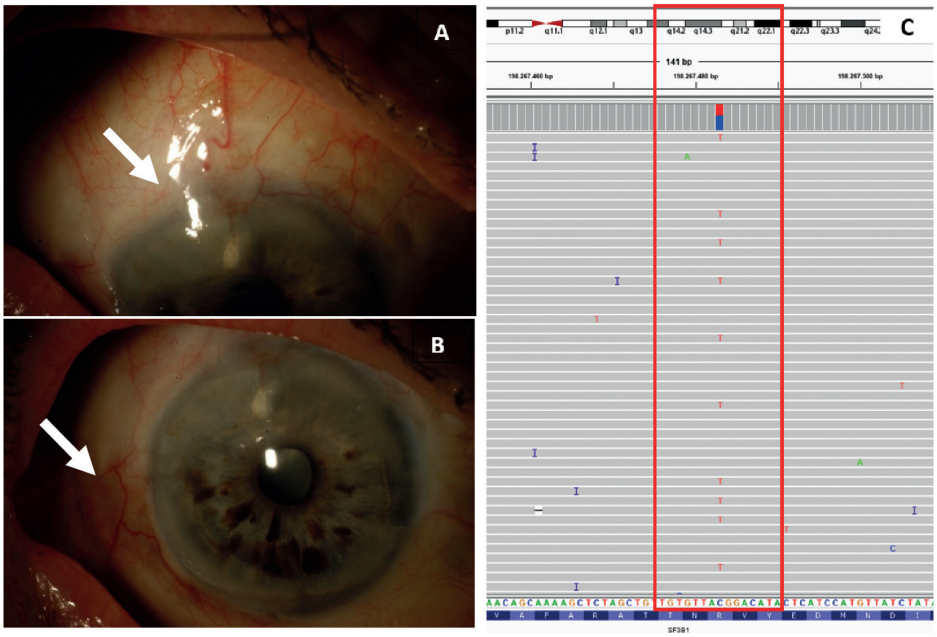

Figure 1. Clinical pictures and molecular data concerning the conjunctival melanoma harboring a *SF3B1* mutation. **A)** Macroscopic view of the melanoma located on the bulbar conjunctiva. **B)** Primary acquired melanosis with atypia component (white arrow). **C)** Depicted in the red box is the molecular data concerning a p.Arg625His mutation in *SF3B1*, with an allele frequency of 42%, using the Integrative Genomics Viewer.

The metastasis-free survival (MFS) of patients with a *TERT* promoter mutation was significantly shorter as compared to patients without a *TERT* promoter mutation in the tumor ($p = 0.008$, Table 2, Figure 2). No correlation between metastasis-free survival and mutation status of *BRAF*, *BAP1*, *SF3B1*, *NRAS*, *c-KIT*, and *PTEN* could be observed.

Table 2. Presence of a mutations versus metastasis-free survival (MFS) and recurrence-free survival (RFS)

Gene	Presence of a mutation	n (%)	Metastasis n (%)	MFS p-value	Recurrences n (%)	RFS p-value
SF3B1				0.45		0.45
	Yes	1 (4)	0 (0)		0 (0)	
	No	27 (96)	9 (33)		8 (30)	
BAP1				0.46		0.69
	Yes	5 (18)	1 (20)		2 (40)	
	No	23 (82)	8 (35)		6 (26)	
TERT				**0.008**		0.20
	Yes	15 (54)	7 (47)		2 (13)	
	No	13 (46)	2 (15)		6 (46)	
NRAS				0.17		0.82
	Yes	6 (21)	4 (67)		2 (33)	
	No	22 (79)	5 (23)		6 (27)	
KIT				0.26		0.88
	Yes	2 (7)	0 (0)		1 (50)	
	No	26 (93)	9 (35)		7 (28)	
PTEN				0.53		0.25
	Yes	4 (14)	1 (25)		2 (50)	
	No	24 (86)	8 (33)		6 (25)	
BRAF				0.052		0.76
	Yes	13 (46)	5 (38)		2 (15)	
	No	15 (54)	4 (27)		6 (40)	

The total number of included conjunctival melanoma cases was twenty-eight. This table depicts the percentages of the specific mutations in the cohort, as well as the development of metastatic disease and recurrent disease within the group of a specific mutation. The statistically significant p-value is depicted in bold. MFS = metastasis –free survival; RFS = recurrence-free survival.

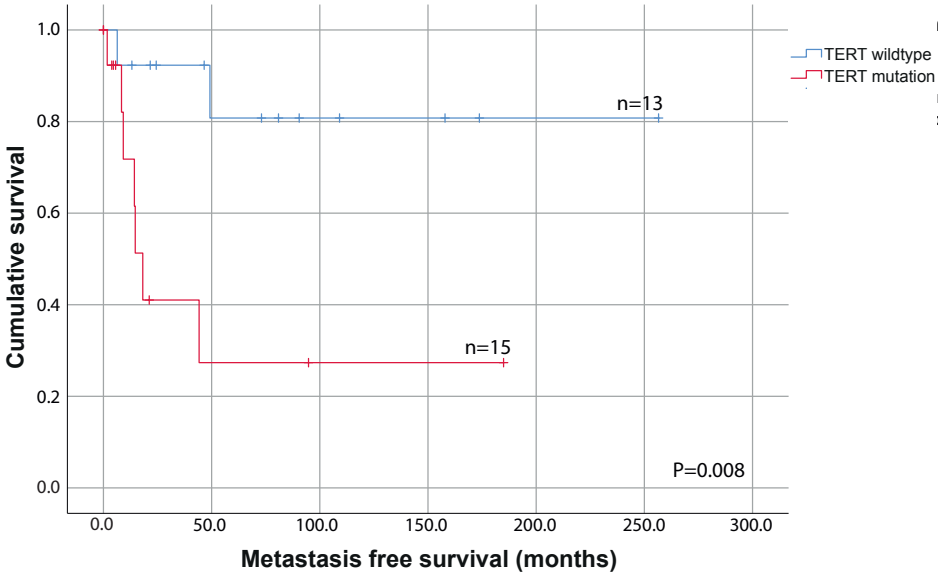

Figure 2. Kaplan–Meier survival estimate for the presence of a TERT promoter mutation in conjunctival melanoma. Kaplan– Meier survival estimate for the time to metastasis of conjunctival melanoma (CM), showing that patients with a CM with a TERT promoter mutation are more likely to develop metastatic disease

No correlation was found between the presence of any mutations and the development of recurrences (Table 2). We also analyzed whether the mutations were correlated with sex, age, location (bulbar only versus involvement of the palpebral/caruncular/forniceal conjunctiva), pT status (pT1 versus pT2), tumor thickness, origin (PAM-derived melanoma versus non-PAM-derived melanoma). We did find an association between the presence of a *TERT* promoter mutation and the origin of the lesion (*p*-value = 0.005), with most cases (54%) developing either de novo or from a melanocytic nevus (Table 3).

Immunohistochemistry

In five CM cases that revealed a *BAP1* mutation using molecular testing, there was enough material available for testing the presence of a *BAP1* mutation using immunohistochemistry. Four of these cases did not show loss of expression of *BAP1* using immunohistochemistry, while one CM case did show loss of expression using *BAP1* immunohistochemistry, with presence of positive (internal) control tissue.

Table 3. Mutations versus clinical and histopathological parameters

	TERT n=15 (%)	p	*BRAF* n=13 (%)	P	*BAP1* n=5 (%)	P	*NRAS* n=6 (%)	p	*PTEN* n=4 (%)	P	*c-KIT* n= 2 (%)	p	*SF3B1* n=1 (%)	P
Gender		0.26		0.71		1.00		1.00		1.00		1.00		1.00
Male	6 (40)		6 (46)		3 (60)		3 (50)		3 (75)		1 (50)		1 (100)	
Female	9 (60)		7 (54)		2 (40)		3 (50)		1 (25)		1 (50)		0 (0)	
Age		0.91		0.91		0.52		0.32		0.92		0.24		0.50
<50y	2 (13)		2 (15)		0 (0)		2 (33)		0 (0)		0 (0)		0 (0)	
50-65y	7 (47)		6 (46)		3 (60)		2 (33)		2 (50)		2 (100)		1 (100)	
>65y	6 (40)		5 (38)		2 (40)		2 (33)		2 (50)		0 (0)		0 (0)	
Location		0.16		0.85		1.00		1.00		0.59		1.00		0.48
Bulbar	8 (53)		6 (46)		2 (40)		2 (33)		1 (25)		1 (50)		1 (100)	
Forniceal/ palpebral/ caruncular involvement	5 (33)		6 (46)		2 (40)		2 (33)		2 (50)		1 (50)		0 (0)	
Tumor thickness		0.67		0.68		1.00		0.63		0.56		0.53		0.31
Tumor thickness ≤2 mm	5 (33)		3 (23)		1 (20)		2 (33)		2 (50)		1 (50)		1 (100)	
Tumor thickness >2mm	8 (53)		9 (69)		4 (80)		3 (50)		2 (50)		1 (50)		0 (0)	
pT status		0.16		0.85		1.00		1.00		0.59		1.00		0.48
pT1	8 (53)		6 (46)		2 (40)		2 (33)		1 (25)		1 (50)		1 (100)	
pT2	5 (33)		6 (46)		2 (40)		2 (33)		2 (50)		1 (50)		0 (0)	
Origin		**0.01**		1.00		1.00		0.30		1.00		1.00		1.00
PAM	6 (40)		7 (54)		3 (60)		3 (50)		3 (80)		2 (100)		1 (100)	
Non PAM (nevus/ de novo)	7 (47)		3 (23)		1 (20)		3 (50)		1 (25)		0 (0)		0 (0)	

DISCUSSION

Pathways involved in the pathogenesis of CM included the MAPK/ERK pathway and the PI3K/AKT pathways, and these pathways overlap with the pathways involved in cutaneous melanoma.[6] The mutation that we found most frequent in CM is a *TERT* promoter mutation, congruent with other studies concerning ocular melanoma[6,13,14] and cancer originating from other sites. These mutations result in a new consensus binding site for E-twenty-six (ETS) transcription factors and this may contribute to increased TERT. The ETS transcription factors are downstream targets of the RAS-RAF-MAPK pathways, and *TERT* promoter mutations are suggested to have synergistic effects with activating *BRAF* or *NRAS* mutations to promote tumor cell proliferation.[21] *TERT* is involved in the AKT pathway, and plays an important role in cellular immortality.[6] *TERT* mRNA overexpression does not completely explain all effects of the *TERT* promoter mutations in tumorigenesis, and the role of immunohistochemistry in determining the *TERT* status is still a topic of debate.[22] Consequently, other undefined or epigenetic mechanisms of *TERT*-upregulating are expected to exist.[21,23,24] While a *TERT* promoter mutation is not found in conjunctival nevi, it is found in both PAM[14] and CM[6,14] with increased *TERT* expression leading to tumor progression.[6] In this context, the C>T or CC>TT nucleotide changes in these mutations are of interest, since this is the typical UV signature, in line with the UV-exposed location of most CM, as seen in our study and as compared to the molecular make up of cutaneous melanoma.[6] UM usually do not harbor mutations in or near the *TERT* gene.[14,18,25] It indicates that different pathways are involved in the development of CM and UM, as is also suggested by the differences in the presence of mutations in *BRAF*, *NRAS*, and *GNAQ*/*GNA11*.

Since *TERT* promoter mutations are relatively common in CM, these mutations are of special interest with respect to clinical consequences. We did not find a correlation between the presence of any of the investigated mutations in this study and the well-known adverse histopathological parameters, as has been described for cutaneous melanoma, such as increasing tumor thickness and more advanced pT stage.[26] Previous studies reported an association between PAM with atypia and PAM-derived melanoma, with the presence of a *TERT* promoter mutation.[13,14] Remarkably, in the current study, we found a significant association with the presence of a *TERT* promoter mutation and non PAM-derived melanoma. This difference needs to be clarified by testing larger cohorts. The presence of a *TERT* promoter mutation in the tumor could have important clinical consequences, including the correlation of mutation status of this gene and follow-up. We found a correlation between the presence of a *TERT* promoter mutation and MFS, with a lower MFS in patients with a CM with a *TERT* promoter mutation, congruent with the findings in our previous study.[13] *TERT* promoter mutations have also been described as an independent prognostic

factor in cutaneous melanoma. From this perspective, it is important to mention that most lesions in our cohort concerned relatively large tumors located at prognostic adverse locations (palpebra, fornix, or caruncle),[6] suggesting a bias. Patients with a *TERT*-promoter-mutated CM might benefit from an intensified follow-up program.

In addition to *TERT* promoter mutations, CM frequently harbors *BRAF* mutations, which are known to activate the downstream kinases MEK1/2 and ERK1/2, resulting in tumor proliferation.[1,6] In this study, we identified *BRAF* mutations in almost half the cases, almost all resulting in V600E mutations. This is in line with the literature in which 30–40% of all CM harbor mutations in *BRAF*, almost all being V600E mutations.[3,6,13,27,28] These mutations, and specifically the V600E mutation, are also present in about half of all patients with cutaneous melanoma,[29] whereas this mutation is not frequently involved in other mucosal melanoma or UM.[6]

In cutaneous melanoma, the presence of a *TERT* promoter mutation in addition to a *BRAF* mutation is associated with unfavorable clinicopathological characteristics, such as large tumor thickness and a high mitotic rate.[26] Unfortunately, the number of cases in the current cohort was too small to render any conclusions concerning these correlations in CM.

Determining the mutation status of the tumor could be useful with regards to therapeutic consequences, since several studies have shown an improved progression-free survival and overall survival, in patients with metastasized cutaneous melanoma harboring a *BRAF* mutation, using BRAF inhibitors.[30] *BRAF* mutations are also attractive as a target for adjuvant therapy in CM.[6,31-33]

NRAS mutations are described in 27% of cutaneous melanoma, with a Q61K mutation as the most common mutation followed by Q61R.[34] *NRAS*-mutated cutaneous melanoma have an unfavorable prognosis as compared to *BRAF* mutated or wild-type melanoma.[34] We identified *NRAS* mutations in 21% of all CM in our cohort, which is in line with the 17% previously reported[15] and is somewhat lower compared to other literature.[6] Due to the small numbers of *NRAS*-mutated cases in our cohort, no correlations to prognosis could be determined. *NRAS* mutations are mutually exclusive with *BRAF* mutations.[6] *NRAS* mutations are amenable to *MEK* inhibitor therapy, as has been shown for cutaneous melanoma.[35] *MEK* inhibitors reduce the growth of *NRAS* mutant CM cell lines.[1] As yet, no cases of *NRAS*-mutated metastatic melanoma treated with *MEK* inhibitors have been published.

Interestingly, we detected an *SF3B1* mutation at the hotspot R625, which is well-known in UM,[3,28] and was reported in one CM case. The presence of a *SF3B1*

mutation was reported previously in CM, however, this concerned a p.C1123Y mutation and not a hotspot mutation,[36] and another study reported a missense mutation.[15] Although R625 *SF3B1* mutations are very rare in most melanoma, they have been identified in UM, including iris melanoma,[19] and are less frequent in cutaneous melanoma as well as in vulvovaginal mucosal melanoma.[36-39] The occurrence of *SF3B1* mutations in mucosal melanoma other than CM is higher, with a prevalence of 42% and hotspot mutations in 30–37%.[39,40] The clinical significance of this mutation in CM is unknown, whereas in UM, *SF3B1* mutation is correlated to late metastatic disease.[41] The CM with this mutation was treated with excision. This case also included PAM and showed local recurrence, three and eight years after primary treatment. No metastasis developed in the follow-up period of 6.8 years. However, metastasis in *SF3B1*-mutated UM was described even after 10 years.[41]

The CM cases in our cohort also harbored mutations in c-*KIT*, *PTEN*, and *BAP1*. These findings of mutations in c-*KIT*, *NRAS*, and *PTEN* are congruent with other literature,[1,6] with c-*KIT* mutations reported in 39% of mucosal melanoma and being feasible for targeted therapy.[42] Of interest is the finding of mutations in *BAP1*, which is a common hemizygous mutation in UM.[12,43] *BAP1* is a tumor suppressor gene and individuals with cutaneous melanocytic neoplasm with a germline *BAP1* mutation, often have *BRAF* mutations, with these lesions reported to have a benign clinical course.[43] However, UM with somatic *BAP1* mutations are correlated to loss of chromosome 3 and early metastatic disease. CM has also been described in a patient with the *BAP1* tumor predisposition syndrome.[44] We identified heterozygous *BAP1* mutations that can be explained as passenger mutations without consequences, due to expression of the remaining non-affected allele.

The genetic profile of CM differs from UM, another subtype of ocular melanoma, in which mutations in *GNAQ*/*GNA11* are frequently described.[45] In this study, none of the CM harbored an activating hotspot mutation in *GNAQ* or *GNA11.* These findings are congruent with other studies analyzing mutations in CM.[15,46] *BRAF* and *NRAS* mutations are extremely rare in UM.[37] Therefore, these mutations can be useful in distinguishing CM from UM. This may be of interest in the identification of the primary tumor site in the case of metastatic melanoma with unknown primary. It also warrants the need for exploration of the genetic background of metastatic melanocytic lesions. However, such molecular results need to be interpreted with care, since we describe *BAP1* and *SF3B1* mutations in CM in the current cohort.

We did not find a correlation concerning the presence of any of the mutations and the development of recurrent disease. Cases with recurrent disease harbored the most frequently found mutations only in a (very) low number

of cases. This may imply that recurrence and metastasis relate to different molecular or physical processes.

In conclusion, based on our molecular findings, CM comprises a separate entity within the ocular melanoma group, although there certainly are overlapping molecular features with UM, such as the presence of *BAP1* and *SF3B1* mutations. This warrants careful interpretation of molecular data in the light of clinical findings. About three-quarter of CM contain drug-targetable mutations in *BRAF*, *NRAS*, or *c-KIT*, supporting the relevance of molecular genetic testing in CM for therapeutic reasons. Within this study, we confirmed that *TERT* promoter mutations are frequently found in CM and are correlated to metastatic disease, supporting the relevance of molecular genetic testing for prognostic reasons.

MATERIALS AND METHODS

Material Selection

We collected twenty-eight CM, diagnosed between 1987 and 2016 at the Erasmus MC University Medical Center (Rotterdam, The Netherlands) and The Rotterdam Eye Hospital (Rotterdam, The Netherlands). Ethics Committee approval was obtained by the Medical Ethics Committee, Erasmus MC-University Medical Center, Rotterdam, The Netherlands (4 Oct 2018) and was registered with reference 67865. The study was performed according to the tenets of the Declaration of Helsinki. Samples were included when sufficient FFPE material was available for testing. Data regarding gender, age at the time of diagnosis, location, tumor thickness, the origin of the lesion, and information of development of recurrences and metastasis were collected from the patient records and information was obtained from the pathology reports and the nationwide-pathology network and registry system (*Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief*). Recurrence was defined as histopathological proven CM at the same location, either after complete excision of the primary lesion or a tumor-free mapping biopsy, after a first incomplete excision of the primary tumor. Recurrence-free survival was defined as the time from the primary treatment to the date of recurrence or last date of follow-up. Metastasis-free survival was defined as time from the primary treatment to the date of metastatic disease or last date of follow-up.

DNA Isolation

DNA from FFPE tissue was isolated using lysis buffer (Promega, Madison, WI, USA) and 5% Chelex (Bio-Rad, Hercules, CA, USA), as described previously[27] and stored at −20 °C. DNA concentrations were measured with the Quant-iT™ PicoGreen™ ds DNA Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Targeted Next-Generation Sequencing

The Ion Personal Genome Machine and Torrent Server (Thermo Fisher Scientific, Waltham, MA, USA) was used for targeted next-generation sequencing (NGS), according to the manufacturer's protocol. An input of DNA was used depending on the available amount of DNA. An extended gene panel covering *GNAQ*, *GNA11*, *EIF1AX*, *SF3B1*, *BAP1*, *BRAF*, *NRAS*, *c-KIT*, *PTEN*, and *TERT* was used, as described previously.[27]

Mutation Analysis

Mutation analysis was performed independently by an ophthalmology resident (NvP) and a fellow in ophthalmic pathology (JvI), trained in the evaluation of NGS data. All data were analyzed manually using Integrative Genomics Viewer (IGV) Version 2.3.68 (97) (Broad Institute, Cambridge, MA). Furthermore, an automatic filtering of the variant calling files (vcf) was done according to the following criteria: inclusion of the hotspots at *GNAQ*/*GNA11* (R183 and Q209) and *SF3B1* (R625), and other variants meeting the following criteria: coverage of at least 50 reads and an allele frequency of at least 10%. Single nucleotide pleomorphisms (SNP's), synonymous, intergenic, and intronic variants were excluded, but intronic variants with possible splice effects were scored. Subsequently, the filtered mutations were verified using IGV (Broad Institute, Cambridge, MA, USA), and compared to the mutations that were detected manually.

Immunohistochemistry

The presence of a mutation in the *BAP1* gene was also evaluated using *BAP1* immunohistochemistry, clone sc-28383, 1:50 dilution (Santa Cruz Biotechnology, Dallas, TX, USA). The samples were scored through masked screening, by an experienced ophthalmic pathologist (RVE).

Survival Analysis

All statistical analysis was performed using IBM SPSS Statistics Version 25 (IBM, Armonk, NY, USA). Kaplan Meier estimates were used to compare survival between groups. Log-rank test was used to test the null hypothesis that there was no difference in survival. A p-value < 0.05 was considered to be statistically significant. For the purpose of analyzing age related to the mutation, age was categorized into three groups: <50 years, 50–65 years, >65 years, analogous to other literature [28]. Fisher's exact test was used to analyze whether a specific mutation was correlated with a specific clinical or histopathological parameter.

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Supplementary Table 1. Overview of genetic mutations

	SF3B1	*BAP1*	*TERT*	*NRAS*	*KIT*	*PTEN*	*BRAF*
1	wt	wt	wt	wt	wt	c.675T>G: p.(Tyr225*)	c.1799T>A: p.(Val600Glu)
2	wt	wt	c.-146C>T	wt	wt	wt	c.1799T>A: p.(Val600Glu)
3	wt	wt	wt	wt	wt	wt	wt
4	wt	wt	wt	wt	wt	wt	wt
5	wt	wt	c.-146C>T	wt	wt	wt	c.1799T>A: p.(Val600Glu)
6	wt	wt	c.-146C>T	wt	wt	wt	c.1799T>A: p.(Val600Glu)
7	wt	wt	wt	wt	wt	wt	wt
8	wt	wt	c.-146C>T	wt	wt	wt	c.1799T>A: p.(Val600Glu)
9	wt	wt	wt	c.181C>A: p.(Gln61Lys)	wt	wt	wt
10	wt	wt	c.-124C>T	wt	wt	c.47dup: p.(Tyr16*)	c.1799T>A: p.(Val600Glu)
11	wt	wt	wt	wt	wt	wt	c.1799T>A: p.(Val600Glu)
12	wt	wt	c.-146C>T	c.181C>A: p.(Gln61Lys)	wt	wt	wt
13	wt	c.1202_1203del: p.(Tyr401*)	c.-146C>T	c.182A>G: p.(Gln61Arg)	wt	wt	wt
14	wt	wt	c.-138C>T c.-139C>T	c.182A>G: p.(Gln61Arg)	wt	wt	wt
15	wt	wt	c.-146C>T	wt	wt	wt	c.1799T>A: p.(Val600Glu)
16	wt	wt	wt	wt	wt	wt	c.1798G>A + c.1799T>A :p.(Val600Lys)
17	wt	c.1144G>A: p.(Gly382Ser)	wt	wt	c.1144G>A: p.(Gly382Ser) c.2435G>T: p.(Gly812Val)	c.133G>A: p.(Val45Ile) c.701G>A: p.(Arg234Gln)	wt

Supplementary Table 1. Continued.

	SF3B1	*BAP1*	*TERT*	*NRAS*	*KIT*	*PTEN*	*BRAF*
18	wt	wt	wt	wt	wt	wt	wt
19	wt	wt	c.-146C>T	wt	wt	wt	c.1799t>a: p.(val600glu)
20	wt	wt	c.-113C>T c.-124C>T	wt	wt	wt	wt
21	wt	wt	wt	wt	wt	wt	wt
22	c.1874G>A:p.(Arg625His)	wt	wt	wt	c.1697_1720del:p.(Asn566_Pro573del)	wt	wt
23	wt	wt	c.-146C>T	wt	wt	wt	c.1799T>A: p.(Val600Glu)
24	wt	wt	c.-146C>T	wt	wt	wt	c.1799T>A: p.(Val600Glu) c.1362T>A:p.(Asp454Glu)
25	wt	wt	c.-146C>T	c.183A>T:p.(Gln61His)	wt	wt	wt
26	wt	c.1729+2T>A	wt	c.34G>C:p.(Gly12Arg)	wt	wt	wt
27	wt	c.1208A>G:p.(Asp403Gly)	c.-146C28>T	wt	wt	wt	c.1796_1797insCAT:p.(Thr599_Val600insIle)
28	wt	c.935G>A:p.(Gly312Asp)	wt	wt	wt	c.634+1G>A	wt

Chapter 2.3

Chromosomal rearrangements in uveal melanoma: chromothripsis

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ABSTRACT

Uveal melanoma (UM) is the most common primary intraocular malignancy in the Western world. Recurrent mutations in *GNAQ*, *GNA11*, *CYSLTR2*, *PLCB4*, *BAP1*, *EIF1AX*, and *SF3B1* are described as well as non-random chromosomal aberrations. Chromothripsis is a rare event in which chromosomes are shattered and rearranged and has been reported in a variety of cancers including UM. SNP arrays of 249 UM from patients who underwent enucleation, biopsy or endoresection were reviewed for the presence of chromothripsis. Chromothripsis was defined as ten or more breakpoints per chromosome involved. Genetic analysis of *GNAQ*, *GNA11*, *BAP1*, *SF3B1*, and *EIF1AX* was conducted using Sanger and next-generation sequencing. In addition, immunohistochemistry for BAP1 was performed. Chromothripsis was detected in 7 out of 249 tumors and the affected chromosomes were chromosomes 3, 5, 6, 8, 12, and 13. The mean total of fragments per chromosome was 39.8 (range 12-116). In 1 UM, chromothripsis was present in 2 different chromosomes. *GNAQ*, *GNA11* or *CYSLTR2* mutations were present in 6 of these tumors and 5 tumors harbored a *BAP1* mutation and/or lacked BAP1 protein expression by immunohistochemistry. Four of these tumors metastasized and for the fifth only short follow-up data are available. One of these metastatic tumors harbored an *SF3B1* mutation. No *EIF1AX* mutations were detected in any of the tumors. To conclude, chromothripsis is a rare event in UM, occurring in 2.8% of samples and without significant association with mutations in any of the common UM driver genes.

INTRODUCTION

Uveal melanoma (UM) is a relative rare disease and has a high mortality rate due to metastasis in about half of all patients within 15 years after diagnosis.[1-3] It is the most common primary intra-ocular malignancy in adults in the Western world.[4] UM specific mutations in the alpha subunit genes *GNAQ* and *GNA11* are described as well as mutations in *BAP1*, *SF3B1* and *EIF1AX*.[5-7] Mutations in the latter three genes are found in approximately 75% of all UM and are useful for prognostication of patients.[8-10] *BAP1*-mutated UM gives rise to early-onset metastasis whereas *SF3B1*-mutated UM gives rise to late-onset metastasis and *EIF1AX*-mutated UM hardly metastasizes.[8] Mutations in *PLCB4* and *CYSLTR2* are described in UM in a mutually exclusive manner to *GNAQ* or *GNA11* mutations but so far have not been associated with prognosis.[11, 12] Copy number alterations in chromosomes 1, 3, 6 and 8 are correlated with prognosis of the UM patient.[13, 14] UM with *EIF1AX*, *SF3B1* and *BAP1* mutations are associated with unique chromosomal patterns, suggesting distinct UM subclasses. *BAP1*-mutated UM harbors entire chromosome copy number variations (CNVs) and entire chromosome arm CNV anomalies (isochromosomes). UM with an *SF3B1* mutation is characterized by many structural variants, often affecting the terminal ends of chromosomes and thus not entire chromosomes or chromosome arms.[15] Besides these recurrent CNVs, also other cytogenetic patterns are described such as polyploidy of the genome, which occurs in approximately 10-15% of all UM.[16] Another chromosomal anomaly described in UM is chromothripsis.[14] This is a phenomenon in which many genomic rearrangements occurs in a single chromosome or chromosome arm. It has been described in congenital abnormalities, UM and a variety of other cancers such as bone cancer, lung cancer, myelodysplastic syndrome (MDS), colorectal cancer, breast cancer and neuroblastoma.[14, 17-21] Chromothripsis predicts a poor outcome in skin melanoma and occur in high risk neuroblastoma, breast cancer and MDS.[18, 20-22] A positive correlation between chromothripsis and progression free survival was observed in metastatic colorectal cancer.[19] The clinical consequence of this phenomenon in UM remains unclear.[14] In this case series we report on chromothripsis in 7/249 UM.

The mechanism of chromothripsis remains elusive but several hypotheses are described such as the formation of micronuclei, premature chromosome compaction (PCC), *TP53* mutations and breakage-fusion bridge cycles or irradiation.[23-25] The formation of chromothripsis involving telomere regions and one chromosome arm is described and supports the hypothesis that events during the cell cycle are involved in the formation of these chromosomal rearrangements.[26] It is hypothesized that chromothripsis occurs through the formation of micronuclei that arise from lagging chromosomes or chromatid fragments during mitosis.[17, 27-30] Moreover, these micronuclei are more prone

to DNA damage, with subsequently DNA nuclease repair by non-homologous end joining (NHEJ), which could explain the chromosome reshuffling.[17, 27, 30, 31]

MATERIALS AND METHODS

Inclusion

Patients with UM that underwent enucleation, endoresection or tumor biopsy at the Erasmus University Medical Center (Rotterdam, The Netherlands) or The Rotterdam Eye Hospital (Rotterdam, The Netherlands) between 1992 and 2017 were selected. SNP (single nucleotide polymorphism) array data of the tumor were available from 249 patients. Chromothripsis was defined as ten or more breakpoints per chromosome detected with SNP array. A breakpoint is present between two fragments with different copy number states in a chromosome. This study was approved by the local ethics committee and followed the tenets of the Declaration of Helsinki. Informed consent was obtained prior to treatment.

SNP array

DNA was extracted from fresh tumor samples using the QIamp DNA-mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. SNP-array was performed using 200 ng of DNA as input for whole-genome analysis (Illumina, San Diego, CA). The data were analyzed with Nexus Copy Number 9.0 software (BioDiscovery Incorporated, El Segundo, CA). The amount of copy number gains and losses was used to determine the number of fragments. The total fragments were counted including copy number neutral fragments as separate fragments.

Mutation detection

Mutation analysis of *GNAQ, GNA11, EIF1AX, SF3B1* and *BAP1* was performed with Sanger sequencing and Ion Torrent next-generation sequencing (NGS) (Thermo Fisher Scientific, Waltham, MA) as described before.[32] UM without a *GNAQ* or *GNA11* mutation were sequenced for *PLCB4* and *CYSLTR2*. If the tumor did not harbor a mutation in *EIF1AX*, *SF3B1* or *BAP1,* mutation analysis for *SRSF2* was performed. SeqScape Software 3 (Applied Biosystems, Foster City, CA) and Integrative Genomics Viewer (IGV) Version 2.3.68 (97) (Broad Institute, Cambridge, MA) was used to analyze the data. BAP1 immunohistochemistry (IHC) was scored for the presence of nuclear BAP1 expression and performed as described previously.[9]

RESULTS

Patient characteristics

Chromothripsis was detected in the UM of seven patients. These comprised five women and two men with a mean age at diagnosis of 57.4 years (range 46.2 – 73.4 years). Six patients underwent enucleation as primary treatment. In one patient, primary treatment was followed by external beam radiotherapy because of unclear surgical margins. One patient underwent brachytherapy as primary treatment, followed by enucleation almost three years later due to tumor recurrence. Metastasis developed in five patients after 31.9 to 78.7 months. In one patient, a metastasis was located subcutaneously in abdominal skin followed by a local relapse in the orbit. Three years later metastases in the liver and bone were detected. One patient developed metastasis in the bone, lung and paramediastinal nodes. Metastases in the liver were present in two patients, together with cutaneous, muscular and retroperitoneal nodal metastases in one of them. In one patient the location of metastases is unknown. The mean disease free survival (DFS) was 51.5 months (range 15.5 – 99.0 months). In Table 1 an overview of patient characteristics is listed. For none of these patients was a family history including UM or other related cancers documented.

Table 1. Overview of clinical and tumor characteristics

Patient	Sex	Age	DFS	Metastasis	Tumor diameter (mm)	Tumor thickness (mm)	Primary treatment
UM 1	F	46.3	42.7	Yes	14	10	Enucleation
UM 2	M	46.2	78.7	Yes	13	N.a.	Enucleation
UM 3	F	57.4	47.4	Yes	9.5	2	Brachytherapy
UM 4	F	64.1	31.9	Yes	14	12	Enucleation
UM 5	M	55.8	99.0	No	12	4	Enucleation
UM 6	F	73.4	15.5	No	13	7.5	Enucleation
UM 7	F	58.6	45.4	Yes	19	9.5	Enucleation

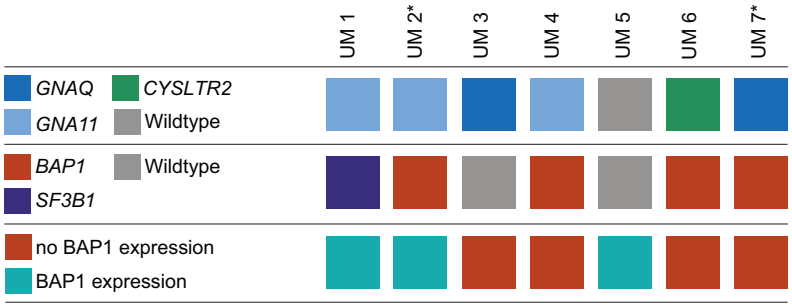
UM = uveal melanoma; F = female; M = male; Age = age at diagnosis in years; DFS = disease free survival in months; N.a. = data not available.

Tumor characteristics

Six tumors were located in the posterior choroid whereas one UM originated from the ciliary body. Mean largest tumor diameter was 13.5 mm (range 9.5-19 mm) and mean tumor thickness 7.5 mm (range 2 – 12 mm) (Table 1). Three UM contained epithelioid cells and four were classified as spindle cell type. Closed vascular loops were present in two of the seven UM and extra-ocular extensions were found in two cases. Inflammatory infiltrate was insignificant in two tumors and present in three tumors, of which extensively in one. Correlations of

chromothripsis with patient and tumor characteristics were not performed due to the limited number of cases.

BAP1 expression was present in three cases and absent in four cases. Mutation analysis was performed in all seven tumors (Figure 1). A mutation in *GNAQ*,c.626A>C:p.(Gln209Pro), was detected in two tumors. A *GNA11* c.626A>T:p.(Gln209Leu) mutation was detected in the UM of three patients. The two UM without a *GNAQ* or *GNA11* mutation did not harbor a mutation in *PLCB4* but in one tumor a c.386T>A:p(Leu129Gln) in *CYSLTR2* was detected (UM 6). One c.1873C>T:p.(Arg625Cys) mutation in *SF3B1* was found (UM 1) but all tumors were wildtype for *EIF1AX*. *BAP1* mutations were detected in four patients: a c.89A>G:p.(Glu31Gly) (UM 2), a c.172_173del:p.(Ser58Profs*10) (UM 6), a c.206_207insA:p.(Thr69Asnfs*5) (UM 7) and a mutation two base pairs after exon 5 (c.375+2T>C) (UM 4) resulting in alternative splicing with a premature stop before the next predicted splice site (prediction in Alamut Visual, Interactive Biosoftware, Rouen, France). Three of these four *BAP1*-mutated UM had an absent BAP1 expression. In one tumor a *BAP1* mutation was not detected with NGS, although IHC revealed lack of BAP1 expression. The two UM without a mutation in *EIF1AX*, *SF3B1* and *BAP1* were wildtype for *SRSF2* as well. Polyploidy occurred in two out of seven UM. See Figure 1 for an overview of mutation status and BAP1 IHC.



	UM 1	UM 2*	UM 3	UM 4	UM 5	UM 6	UM 7*
Chromosome 3 status	D	LOH	M	M	D	M	LOH
Metastasis	Yes	Yes	Yes	Yes	No	No	Yes
Overall survival	3.8	7.9	4.8	2.8	8.2	1.3	8.1
Chromothripsis of chromosome(s)	6	5	3	3	6, 8	12	13
Fragments	24	28	14	27	116, 84	12	13

Figure 1. Overview of patient and tumor characteristics of uveal melanoma with chromothripsis. The first row of blocks represents the mutation status of *GNAQ*and *GNA11*. In UM 5 no mutation in *CYSLTR2* was detected and in UM 5 and UM 6 no mutations in *PLCB4* were found. In the second row of blocks the mutation status of *BAP1* and *SF3B1* is given. None of the UM harbor an *EIF1AX* mutation and UM 3 and UM 6 do not have a mutation in *SRSF2*. The third row of blocks represents the BAP1 IHC staining. Chromosome 3 status of the tumor, whether a patient developed metastasis, the overall survival in years, the chromosome(s) with chromothripsis and the number of fragments per chromosome with chromothripsis are given below. UM = uveal melanoma; D = disomy; M = monosomy; LOH = loss of heterozygosity. *Polyploid tumor.

Chromothripsis

Eight chromosomes showed chromothripsis (Figure 2). One tumor harbored chromothripsis in two separate chromosomes (UM 5; Figure 3). UM 7 (chromothripsis of chromosome 13) showed eight fragments in chromosome 16 as well. However, since this did not meet our criteria of ten fragments, this chromosome was not included for further analysis. Chromosome 3 and 6 were affected in two UM. Regarding chromosome 3, the breakpoints were not present in the *BAP1* gene. Other affected chromosomes were chromosome 5, 8, 12 and 13. The mean of the total fragments per chromosome was 39.8 (range 12-116, Figure 1). In four of the eight chromosomes, the B-allele frequencies indicates more than two copy number states of the separate chromosome fragments (Figure 2A and 3). In five cases (UM 2, UM 3, UM 4, UM 5 and UM 6) DNA from blood was available for germline analysis using SNP array. No chromothripsis was observed in these samples.

DISCUSSION

Recurrent chromosomal aberrations have been described in detail in UM, which are strongly correlated to the mutation status.[15, 33, 34] In this paper, another chromosomal aberration, called chromothripsis, is described. Chromothripsis is characterized by ten to hundreds of chromosome fragments that are shattered and randomly rearranged.[17] This is found in several malignancies with a mean pan-cancer prevalence of 1-2%.[14, 31, 35] Similar to other malignancies, chromothripsis is also rare in UM. In one study chromothripsis was observed in 2/25 UM.[14] We detected chromothripsis in 2.8% of the UM which is in line with the low frequency rate as previously described.

A relation between prognosis and chromothripsis has been reported in several studies on different malignancies. In high risk neuroblastoma, breast cancer and MDS, chromothripsis is correlated with a poor outcome while in metastatic colorectal cancer a better progression free-survival has been described.[15,17-19] Probably metastases with chromothripsis respond better to therapy while the metastatic rate is higher in cancers harboring chromothripsis. This might be true in UM as well; however, no standardized treatment for metastatic UM is available yet. When such treatment is available it might be interesting to compare the response to therapy in UM with and without chromothripsis. Metastatic disease was present in five out of seven patients in this report. Four of the metastasizing tumors harbored a *BAP1* mutation and or lacked BAP1 expression and in one tumor an *SF3B1* mutation was present. One of the two patients without metastatic disease did not harbor a *BAP1* or *SF3B1* mutation in the tumor and the IHC showed a positive BAP1 expression while from the other patient (harboring a *BAP1* mutation in the tumor) only short

follow-up data were available (16 months). The overall poor prognosis of this cohort could be explained by the mutations in *BAP1* and *SF3B1* since it is known that mutations in these genes are correlated with a high risk of metastasis.[8, 10] Therefore, there is no indication that chromothripsis itself causes metastatic disease, but it is possible that the rate of *SF3B1* and *BAP1* mutations is higher in UM with chromothripsis.

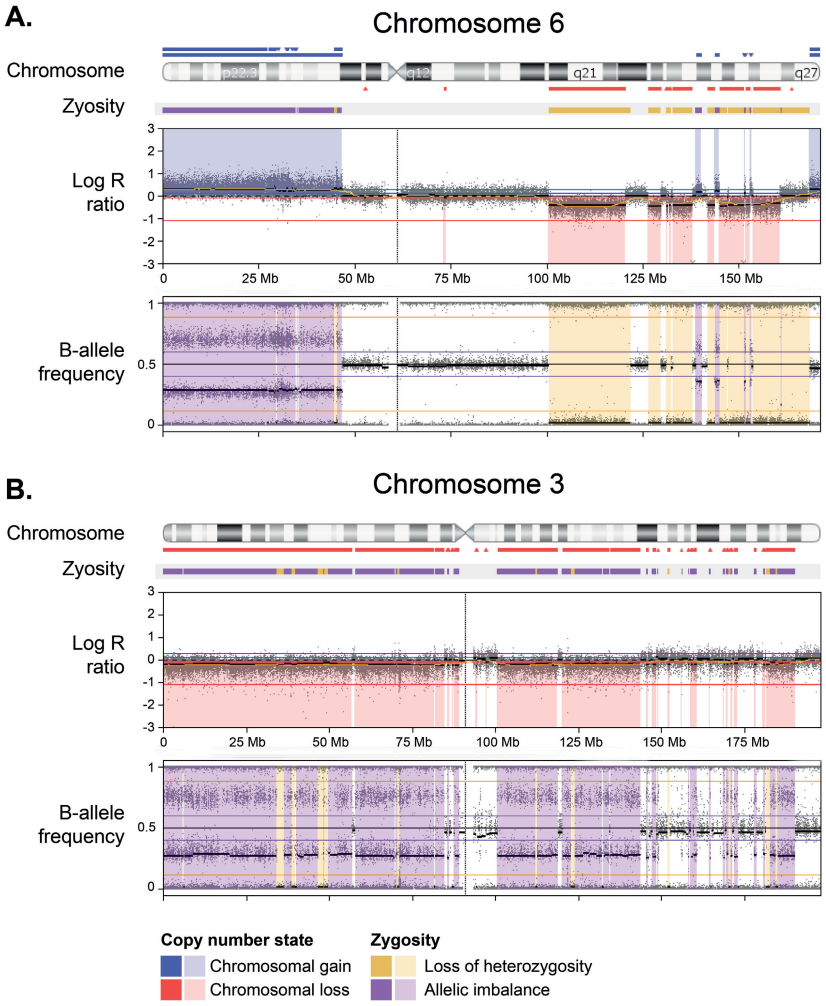


Figure 2. Two examples of chromothripsis. **A)** UM 1 showing chromothripsis of chromosome arm 6q with an additional gain of the terminal short arm of chromosome 6. Note the 3 different copy number states in the chromothriptic chromosome. **B)** UM 4 showing chromothripsis of chromosome 3.

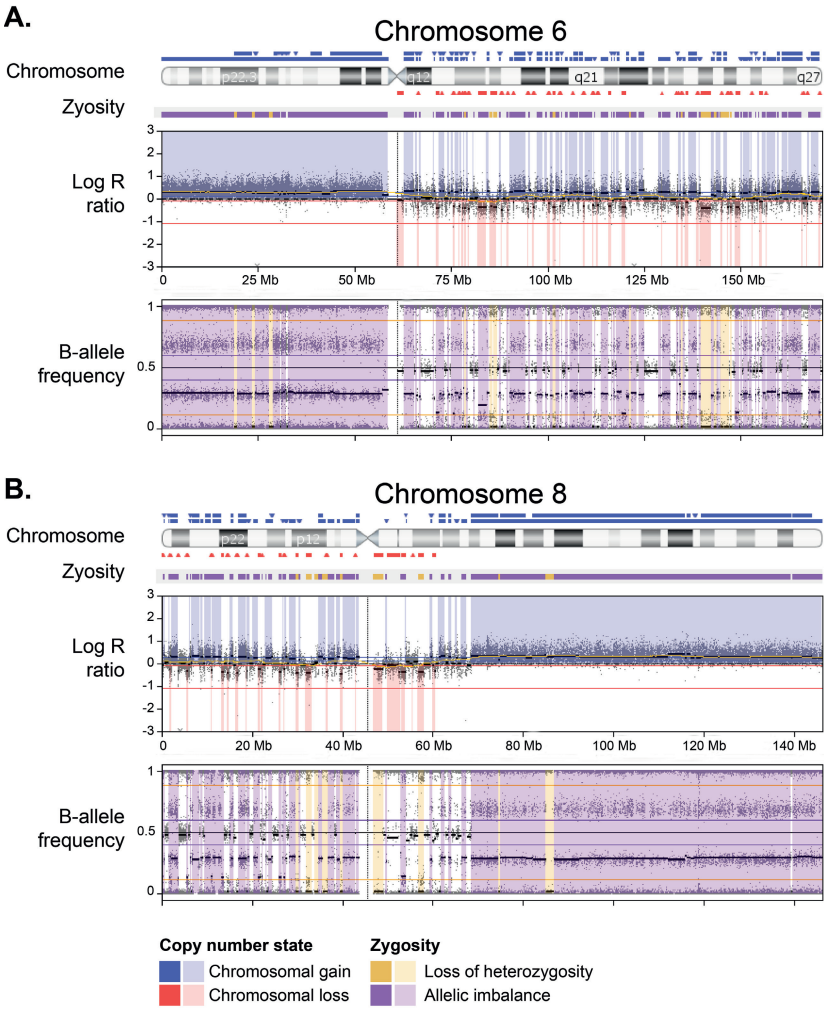


Figure 3. A case with 2 chromothriptic chromosomes. UM 5 showing chromothripsis of **A)** chromosome 6 and **B)** chromosome 8. Note the 3 copy number states and a general gain of the entire chromosomes.

Other features of UM and the relation to chromothripsis could have a clinical impact. In this cohort there were no UM with a hyper mutable status, features of microsatellite instability or an indication of germline mutations causing UM. Consequently, the relation between chromothripsis and these tumor characteristics can not be determined. In about half of the UM, inflammation was present. The tumor with extensive inflammation was the UM with a *BAP1* mutation but without metastatic disease. No conclusions about the immunogenicity can be drawn because of the small numbers of UM with inflammation. Further studies are needed to elucidate these relations and the outcome of patients with chromothriptic UM.

There are several risk factors known for chromothripsis such as irradiation.[23] In one case, brachytherapy was followed by enucleation. Therefore, the chromothripsis in this UM could be an irradiation effect. Other factors correlated with chromothripsis formation are hyper- and polyploidization.[36, 37] For a long time it was assumed that chromothriptic chromosomes only have two copy number states.[17, 30, 31] However, an observation was made in a subtype of acute lymphoblastic leukemia, in which more copy number states were found in chromothriptic chromosomes.[38] In this study, two of the seven UM (29%) were polyploid. Since polyploidy occurs in only 11% of all large UM[16] and chromothripsis is a rare event, this could explain the co-occurrence of polyploid UM with chromothripsis. In addition, in our cohort, seven out of eight chromothriptic chromosomes harbored more than two chromosomes. This observation was also made in the only other study that described two cases of UM with chromothripsis.[14] This suggests that chromothripsis occurs in already duplicated chromosomes. Altered chromosomes might even be more susceptible to chromosome lagging, as 50% of the chromosomes with chromothripsis in this study have more than two copy number states.[38] Furthermore, chromothripsis can occur in more than one chromosome in the same tumor.[17]

In our cohort, more than one chromosome was affected in one tumor. It is noteworthy that the affected chromosomes in this study included chromosomes 3, 6 and 8, since copy number variations in these chromosomes are correlated with mutation status in UM.[15] This is in line with other studies in which chromothripsis occur among known cancer driver genes.[25, 39] Nevertheless, chromothripsis-like patterns across different tumor types showed a limited preference according to chromosome size. However, chromosome 17 was most frequently affected and to a lesser degree chromosomes 8,11 and 12 in another study.[26] This could be explained by the fact that chromosome 17 also harbors *TP53*, an important cancer associated gene, which is correlated to chromothripsis as well.[25]

To conclude, chromothripsis is a complex event that occurs in a variety of cancers.[14, 18, 20, 25, 26, 40] This study shows chromothripsis in almost 3% of UM affecting different chromosomes. Limitation of this study was the small number of cases with chromothripsis. Although a large patient cohort was investigated, the rare occurrence of chromothripsis prohibited proper statistical analyses. Further studies are needed to investigate the evolutionary advantage of this complex chromosomal aberration.

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Chapter 2.4

SRSF2 mutations in uveal melanoma: a preference for in-frame deletions?

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ABSTRACT

Background

Uveal melanoma (UM) is the most common primary ocular malignancy in adults in the Western world. UM with a mutation in SF3B1, a spliceosome gene, is characterized by three or more structural changes of chromosome 1, 6, 8, 9, or 11. Also UM without a mutation in *SF3B1* harbors similar chromosomal aberrations. Since, in addition to *SF3B1*, mutations in *U2AF1* and *SRSF2* have also been observed in hematological malignancies, UM without a *SF3B1* mutation -but with the characteristic chromosomal pattern- might harbor mutations in one of these genes.

Methods

42 UMs were selected based on their chromosomal profile and wildtype *SF3B1* status. Sanger sequencing covering the *U2AF1* (exon 2 and 7) hotspots and *SRSF2* (exon 1 and 2) was performed on DNA extracted from tumor tissue. Data of three UM with an *SRSF2* mutation was extracted from the The Cancer Genome Atlas (TCGA).

Results

Heterozygous in-frame *SRSF2* deletions affecting amino acids 92–100 were detected in two UMs (5%) of 42 selected tumors and in three TGCA UM specimens. Both the UM with an SRSF2 mutation from our cohort and the UM samples from the TCGA showed more than four structural chromosomal aberrations including (partial) gain of chromosome 6 and 8, although in two TCGA UMs monosomy 3 was observed.

Conclusions

Whereas in myelodysplastic syndrome predominantly missense *SRSF2* mutations are described, the observed *SRSF2* mutations in UM are all in-frame deletions of 8–9 amino acids. This suggests that the R625 missense *SF3B1* mutations and *SRSF2* mutations in UM are different compared to the spliceosome gene mutations in hematological cancers, and probably target a different, as yet unknown, set of genes involved in uveal melanoma etiology.

INTRODUCTION

Uveal melanoma (UM) is a primary malignant ocular tumor arising from melanocytes in the uvea which consist of iris, ciliary body, and choroid. Symptoms are present in the majority of patients with the most common presenting symptom being change in vision. Other presenting symptoms include photopsia and floaters.[1] Metastatic disease with predominantly metastasis to the liver, develops in almost half of all UM patients causing a poor prognosis.[1,2] Several prognostic factors are described with mutations in *BAP1*, *SF3B1*, and *EIF1AX,* with or without loss of chromosome 3, as important predictors of survival.[3,4] Tumors of uveal melanoma (UM) patients with somatic *BAP1*, *SF3B1*, or *EIF1AX* mutations show a distinct chromosomal copy number variation (CNV) pattern. Whereas *EIF1AX*^{mut} tumors in general lack gross anomalies, *BAP1*^{mut} tumors display monosomy 3 and isochromosome formation. *SF3B1*^{mut} tumors are characterized by three or more structural variants, usually of chromosomes 1, 6, 8, 9, and 11.[5] However, not all UMs with a typical *SF3B1*^{mut} CNV harbor a mutation in the *SF3B1* component of the spliceosome complex. As in myelodysplastic syndrome (MDS) and MDS-related diseases (such as chronic myelomonocytic leukemia and acute myeloid leukemia) in which mutations in other genes of the spliceosome complex such as *SRSF2* and *U2AF1* are described,[6-10] mutations in *SRSF2* and other spliceosome factors are also observed in UM.[11] Typical MDS-related mutations in *SRSF2* involve codon 95 and are missense mutations resulting in an amino acid change (in 74% of patients with an *SRSF2* mutation) or in-frame deletions starting at this codon (26%).[8] Missense mutations in *U2AF1* in MDS are almost exclusively described in codon 34 (p.Ser34Phe and p.Ser34Tyr), 156 (Arg156His), or 157 (p.Gln157Arg and p.Gln157Pro).[7,12] Therefore, mutation analysis of *SRSF2* and *U2AF1* covering these hotspots was performed on UM tumors with no *SF3B1* mutation but with an *SF3B1*-like chromosomal CNV pattern.

RESULTS

Heterozygous in-frame deletions starting at codon 92 of *SRSF2* were identified in two of the selected 42 UM (p.(Tyr92_His99del); p.(Gly93_His100del)), (Figure 1). These mutations were mutually exclusive for *BAP1*, *SF3B1*, and *EIF1AX* but harbored a *GNAQ* p.(Gln209Leu) mutation (Table 1).

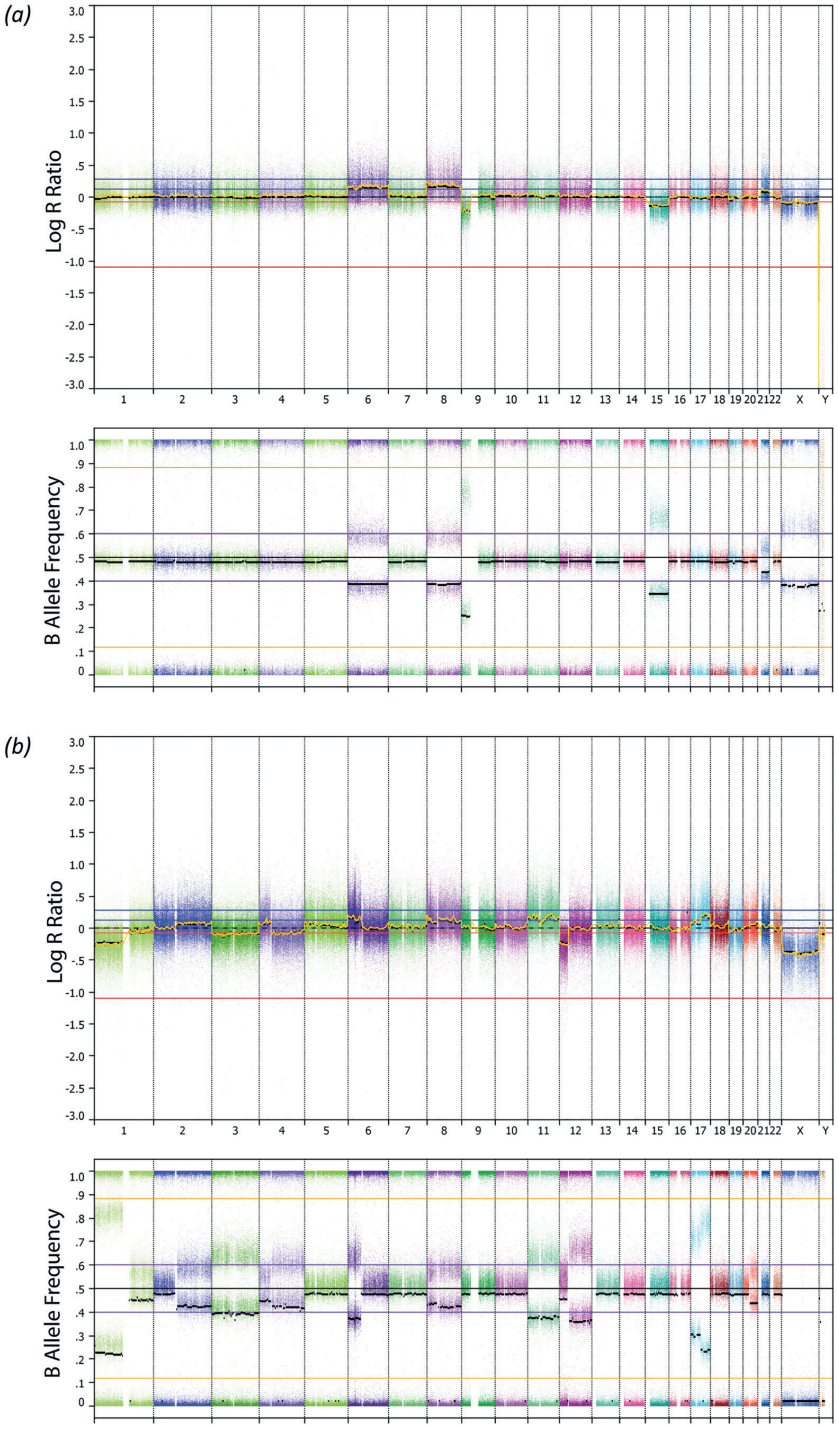

Figure 1. Single nucleotide polymorphism (SNP) array profile with the B-allele frequency from two uveal melanoma samples with an *SRSF2* mutation. On the x-axes the chromosomes are displayed. **A)** Uveal melanoma 1 (UM1). **B)** Uveal melanoma 2 (UM2).

Table 1. Overview of clinical characteristics, mutation status and copy number variation of uveal melanoma (UM) patients with an *SRSF2* mutation

	UM 1	**UM 2**
Clinical characteristics		
Sex	Female	Male
Age at diagnosis (years)	63.0	57.3
Metastasis	No	No
Disease free survival (months)	76.8	128.8
Mutation status		
SRSF2	Chr17(GRCh37):g.74732946_74732969del c.274_297del:p.(Tyr92_His99del)	Chr17(GRCh37):g.74732943_74732966del c.277_300del:p.(Gly93_His100del)
U2AF1	Wildtype	Wildtype
GNAQ	Chr9(GRCh37):g.80409488T>A c.626A>T:p.(Gln209Leu)	Chr9(GRCh37):g.80409488T>A c.626A>T:p.(Gln209Leu)
GNA11	Wildtype	Wildtype
SF3B1	Wildtype	Wildtype
BAP1	Wildtype	Wildtype
EIF1AX	Wildtype	Wildtype
Copy number variation		
(Partial) gain of chromosome	6, 8, 21	2q, 6p, 8, 11, 17, 20q
(Partial) loss of chromosome	9p, 15	1p, 3, 4q, 12p

UM1 originates from the ciliary body and consists of mixed cell type with the presence of closed vascular loops. Largest tumor diameter was 19 mm with a prominence of 8mm. The other UM, UM2, arose from the choroid and consist of spindle cells. No closed vascular loops were present and there was no involvement of the ciliary body. The largest tumor diameter was 13 mm with a prominence of 5 mm with no extraocular extension.

Both UMs showed more than four chromosomal aberrations including gain of chromosome 6 and 8. The single nucleotide polymorphism (SNP) array profiles of these tumors are shown in Figure 2. Both patients did not develop metastatic disease and have a disease-free survival of 76.8 and 128.8 months, respectively. In none of the 42 samples a mutation in *U2AF1* was detected.

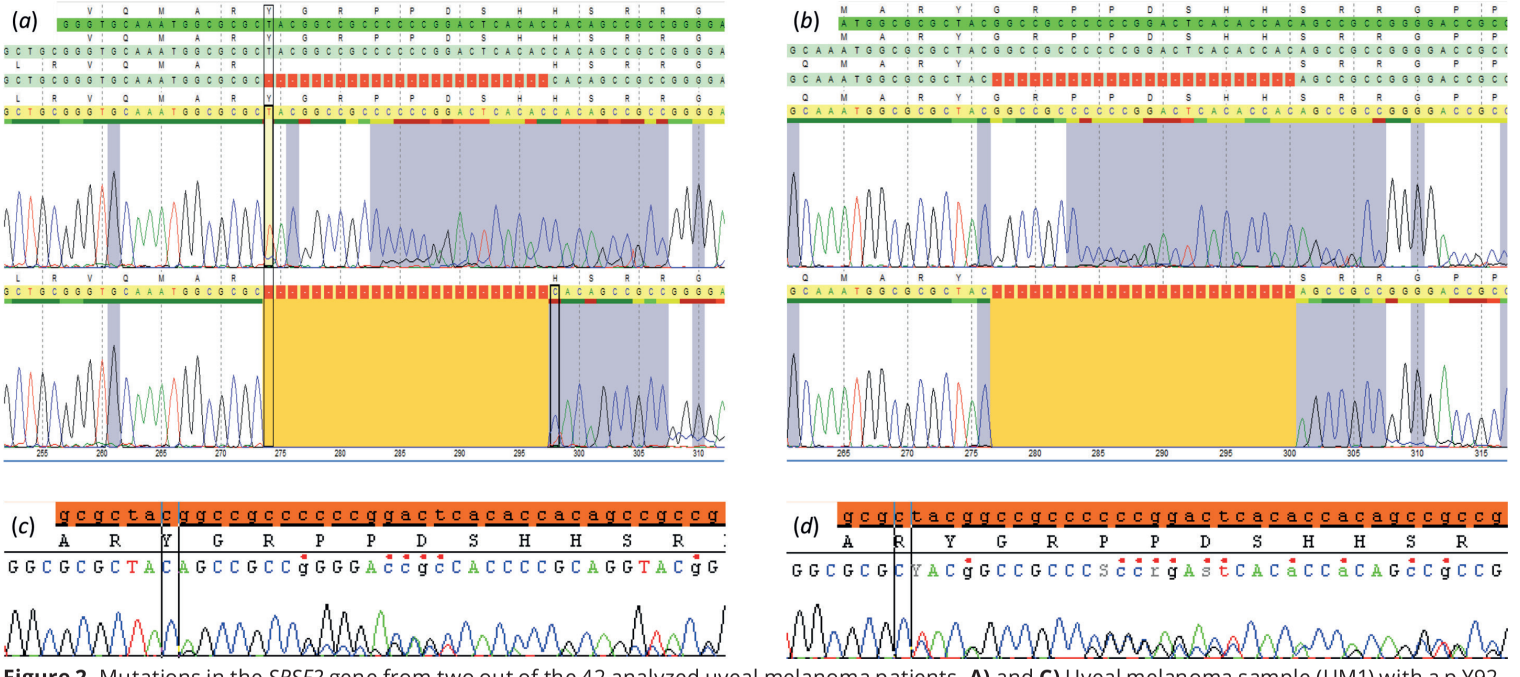

Figure 2. Mutations in the *SRSF2* gene from two out of the 42 analyzed uveal melanoma patients. **A)** and **C)** Uveal melanoma sample (UM1) with a p.Y92_H99del displayed in SeqPilot V4.3.0 (**A**) and in SeqScape V3.0 (**C**). **B)** and **D)** Uveal melanoma (UM2) with a p.G93_H100del displayed in SeqPilot V4.3.0 (**B**) and in SeqScape V3.0 (**D**).

Table 2. Overview of mutations in uveal melanoma samples with an *SRSF2* or *U2AF1* mutation

	SRSF2	*U2AF1*	*GNAQ*	*GNA11*	*BAP1*	*EIF1AX*
1. ROMS	c.274_297del:p.(Tyr92_His99del)	Wildtype	c.626A>T:p.(Gln209Leu)	Wildtype	Wildtype	Wildtype
2. ROMS	c.277_300del:p.(Gly93_His100del)	Wildtype	c.626A>T:p.(Gln209Leu)	Wildtype	Wildtype	Wildtype
3. TCGA	c.274_297del:p.(Tyr92_His99del)	Wildtype	c.626A>T:p.(Gln209Leu)	Wildtype	Wildtype	Wildtype
4. TCGA	c.274_300del:p.(Tyr92_His100del)	Wildtype	Wildtype	c.626A>T:p.(Gln209Leu)	Wildtype	Wildtype
5. TCGA	c.519_536del:p.(Ser174_Ser179del)	Wildtype	Wildtype	c.626A>T:p.(Gln209Leu)	c.518A>G:p.(Tyr173Cys)	Wildtype

ROMS = Rotterdam Ocular Melanoma Studygroup; TCGA = The Cancer Genome Atlas.

Three previously described *SRSF2* mutations were found in the data from the The Cancer Genome Atlas (TCGA) database.[11] CNV analysis showed loss of chromosome 3 in two UMs and gain of chromosome 8(q) in all three UMs. Gain of chromosome 1p was also present in two UMs and gain of chromosome 6 in one sample. Two UMs have a p.(Gln209Leu) mutation in *GNAQ* and one harbors a *GNA11* mutation (p.(Gln209Leu)), (Table 2). No mutations in *EIF1AX* were detected, but one UM has *BAP1* mutation (c.518A > G:p.(Tyr173Cys)).

DISCUSSION

In this study we identified deletions in *SRSF2* in two UM harboring an *SF3B1* specific SNP array pattern albeit with no mutations of the SF3B1 hotspot regions. Studies have shown that in myelodysplastic syndrome (MDS) *SRSF2* was mutated in 12–14% of the cases and mutations in *U2AF1* occur in 15% of the MDS cases.[7,8] This is a higher frequency compared to UM, in which *SRSF2* mutations are detected in less than 5% of the specimens and no *U2AF1* mutations have been identified.[11] Three *SRSF2* mutated UMs described in the literature are included in The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/). Two out of these harbor similar deletions (Table 2) as we have identified in our cohort, and are mutually exclusive with *BAP1* and *EIF1AX*, similar to our own observations. The third *SRSF2* mutation from TCGA is a deletion of amino acid 174–179 and co-exists with a *BAP1* mutation. Surprisingly, this tumor showed a *BAP1* specific CNV profile, indicating that latter deletion of residues 174–179 has no or little pathogenic effect. However, other spliceosome gene mutations can underlie UM pathogenesis but might not display the same chromosomal anomalies as described in *SF3B1*.[5] Furthermore, the low incidence of *SRSF2* mutations in UM suggests that other genes of the splicing machinery, such as *U2AF35* or *ZRSR2,* might be mutated. Mutations in other splicing genes than *SF3B1* could be less frequently involved in the development of UM compared to MDS in which mutations in several splicing genes have been identified.[6,9,10]

Since Sanger sequencing was used for mutation analysis, we have focused on the hotspot regions of *U2AF1* and *SRSF2* that are described in UM and other diseases. More extensive research about mutations in all coding regions of these genes could increase the incidence.

Compared to *SRSF2* mutations in MDS in which the vast majority are missense mutations,[8] we observed a preference for in-frame deletions in UM. Also, for *SF3B1* in UM residue R625 is most commonly mutated residue, whereas in other tumors predominantly the K700 residue of *SF3B1* is affected.[6,11] Thus, although the same gene is involved, mutations occur on different residues in distinct diseases. Furthermore, studying the RNA expression of *SRSF2* mutated UM from

TGCA, we did not observe the same splicing effect as observed in *SF3B1* mutated UM. These findings suggest that *SF3B1* mutations compared to mutations in *SRSF2* have, despite a similar chromosomal pattern, a different effect on splicing.

Since we observed *SRSF2* mutations in only two patients the clinical impact of this mutation remains unclear. However, both patients with an *SRSF2* mutation in our cohort did not develop metastasis within 6 and 10 years, neither did the patients from TCGA. In chronic myelomonocytic leukemia no difference in overall survival was observed, and not in MDS when corrected for age.[8,10] Future studies are needed to evaluate the role of other splicing genes than *SF3B1* in UM.

MATERIALS AND METHODS

Patients with an *SF3B1*-like chromosomal pattern were selected from the Rotterdam Ocular Melanoma Study group (ROMS) database. These UM patients underwent enucleation or biopsy of the tumor in the Erasmus Medical Center (Rotterdam, The Netherlands) or The Rotterdam Eye Hospital (Rotterdam, the Netherlands) between 1993 and 2017. Informed consent from all patients was obtained before collecting the tumor material. This study was performed according to the tenets of the Declaration of Helsinki and approved by the local ethics committee (MEC-2009-375, 12th November 2009).

DNA was isolated from fresh tumor tissue using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and concentrations measured using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Two hundred nanograms of DNA input was used for SNP array analysis using an Illumina Human SNP array platform (Illumina, San Diego, CA, USA). Copy number analysis was performed using Nexus Copy Number 8.0 (BioDiscovery, El Segundo, CA, USA). Moreover, karyotyping was used for CNV analysis when available. Patients were selected from the cohort described previously.[5]

In general, an *SF3B1*-like chromosomal pattern is defined as a combination of three structural variations in SNP array analysis of the tumor (usually this includes either partial gain of chromosome 8q or 9q or partial loss of chromosome 1p or 11q).[5] In addition, UM with gain of chromosome 6p or loss of 6q in addition to one or two other anomalies were also included since these anomalies are also specific for *SF3B1* mutated tumors, whereas this is not seen in *EIF1AX* or *BAP1* mutated UM. Moreover, solely gain of chromosome 6p was only included when the tumor did not harbor an *EIF1AX* mutation, because gain of chromosome 6p is only representative for *SF3B1* and *EIF1AX* mutated UM.

The two coding exons of the *SRSF2* gene were sequenced using Sanger sequencing with primers for these regions (pxlence, Dendermonde, Belgium). The mutation hotspots in *U2AF1* were sequenced with primers covering codon 34, 156, and 157. Sanger sequence results were visualized with SeqScapeSoftware V3.0 (Thermo Fisher Scientific, Waltham, MA, USA) and SeqPilot V4.3.0 (JSI medical systems GmbH, Ettenheim, Germany).

Mutation analysis of *GNAQ*, *GNA11*, *SF3B1, BAP1*, and *EIF1AX* was performed previously using Sanger sequencing and next-generation sequencing using the ION Torrent platform (Life Technologies, Carlsbad, CA, USA).[5,13] A *BAP1* mutation was defined as a mutation in the *BAP1* gene or lack of nuclear BAP1 expression (performed as described previously).[14]

The UM cohort from the National Institute of Health TCGA server (*n* = 80) was used for mutation analysis of *SRSF2* and *U2AF1* using Integrative Genomics Viewer (Version 2.3.68 (97) (Broad Institute, Cambridge, MA, USA). If a mutation in one of these genes was identified, copy number analysis was performed on the segmented SNP array data using Nexus Copy Number 8.0 (BioDiscovery, El Segundo, CA, USA).

CONCLUSIONS

UMs harbor chromosomal aberrations correlated with their mutation status.[5] Mutations in *SF3B1* and *SRSF2*, genes that are both involved in splicing, occur not only in UM but are described in MDS and MDS related diseases as well.[8,11,15] However, the mutation type in these genes are different in both diseases. In UM, *SF3B1* is almost exclusively a missense mutation at residue 625 whereas in other diseases residue 700 is mutated.[6,11] In *SRSF2*, a different type of mutation is also observed in UM compared to MDS and MDS related diseases, but the same region is involved. We identified in-frame deletions of *SRSF2* in UM in the same genetic region, whereas most mutations in the same gene in MDS are missense mutations.[8] Therefore, we conclude that there might be a preference for in-frame deletions in *SRSF2* in UM when this gene is involved. We did not observe any mutation in *U2AF1* in our selected cohort, and the incidence of mutations of *SRSF2* is low. Although we have a selected cohort which might influence the incidence, this is in line with previous studies, in which no or few mutations in these genes are found in UM patients.[11,16,17] The clinical relevance of CNV pattern and the relation to spliceosome mutations remains unclear. More research is needed to evaluate the significance of these findings.

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Chapter 3

Inheritance of uveal melanoma

Chapter 3.1

Germline *BAP1* mutations in uveal melanoma

One of the genes involved in the development of UM is the BRCA1-associated protein-1 (*BAP1*) gene. Somatic mutations in this gene have been described, but it has also been shown that germline mutations can lead to a variety malignant tumors including UM.[1] Families with this so called *BAP1* tumor predisposition syndrome (*BAP1*-TPDS) are described by Walpole et al.[2] This worldwide study, including 181 families, gives an overview of all *BAP1* mutations and correlates genotype and phenotype. Mutation type and age of onset was compared in the different tumor groups regarding missense or null variants. It has been shown that the median age of onset in patients with a null variant was at a younger age compared to those with a missense *BAP1* mutation except for UM and renal cell carcinoma. It is interesting to further elucidate the role of muation type on the development of cancer especially in the germline variants.

Chau et al. showed an overview of families with the *BAP1*-TPDS in the Netherlands.[3] UM was present in nine germline *BAP1* carriers (out of 72 carriers from 22 families) with a median age at diagnosis of 61 years. Co-existing malignancies in UM patients include renal cel carcinoma, mesothelioma and cutaneous melanoma. Surprisingly, also other ocular melanoma such as conjunctival and iris melanoma are described in this cohort. This emphasizes the importance of awareness in patients with a familial history of cancer and the possibility of an underlying genetic predisponance. When looked to the presence of a germline *BAP1* mutation in a cohort of unselected UM patients (n=432), the prevalence was 1.9%.[4] However, in the younger patient group (<50 years), the frequency was higher (3.2%) which also suggests that there is a difference in age of onset between patients with a germline mutation compared to those without a germline *BAP1* mutation.

A guideline for genetic testing based on previous recommendations and the findings of the study from Chau et al. was proposed.[3] This includes the medical and family history and age of onset. Last mentioned differs for the several types of cancer based on the age of onset of these tumors in the population without a germline mutation and those with germline *BAP1* mutations. A schematic overview of these recommendations are shown in the figure below (Figure 1). This guideline is also in line with the national guideline of the Vereniging van Klinische Genetica Nederland (VKGN).[5] In this guideline the age criteria is included as well as the medical history of the index patient and family members. The difference between this guideline compared to the guideline proposed by Chau et al. is that meningioma and cholangiocarcinoma are not included as *BAP1*-associated cancers in case of affected family members. Moreover, an extra criteria including 3rd grade relatives is incorporated. When a patient has UM, cutaneous melanoma, malignant mesothelioma or renal cell carcinoma and a 3rd grade relative with UM, malignant mesothelioma or *BAP1*-inactivaded naevus,

there is an indication for germline testing. Germline testing should always be preceded by counselling and referral to a clinical geneticist.

The vast majority of family members with a germline *BAP1* mutation developed at least one tumor.[2] A difference was noted between the null and missense variant carriers since the null variant carriers were more prone to develop cancer (82.5% vs 60.0%). This indicates the need for proper screening in germline carriers of the *BAP1* gene mutations and the importance to evaluate family history upon patients with a tumor in general but especially one involving in the *BAP1*-TPDS. It is also interesting to look at the risk to develop UM in patients with a germline mutation. The estimated point prevalence, representing the prevalence of UM in *BAP1* germline mutation carriers, is almost 3%.[6] This underlines the importance of ophthalmic examination in this group. The VKGN advices annual ophthalmic examination by an ophthalmologist from the age of 16 years in pathogenic germline *BAP1* carriers.[5] Germline testing should be considered carefully in young individuals with a first degree relative who is a carrier, and can be postponed till adulthood given the potential consequences. Meanwhile, they can be examined annually by ophthalmologists without being tested.

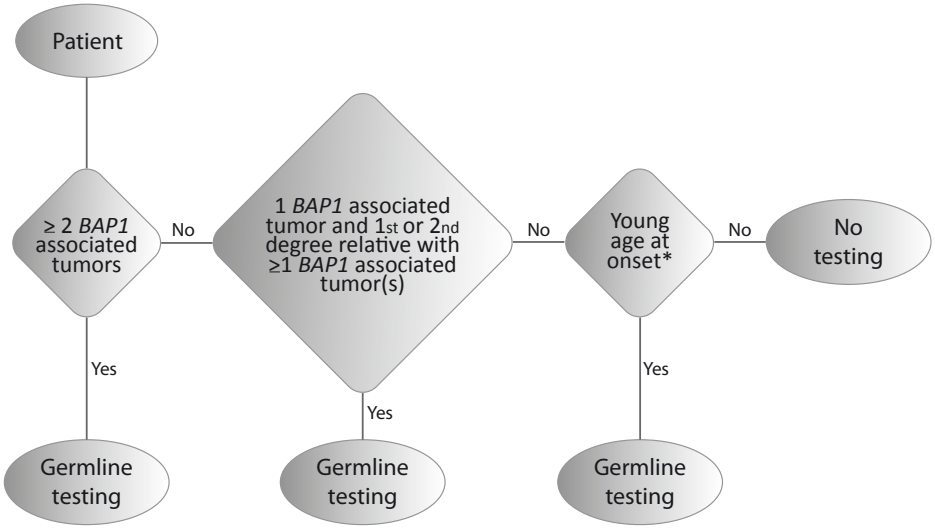

Figure 1. Flowchart of proposed referral guidelines for genetic germline testing.

BAP1-TPDS-associated tumors include: uveal melanoma, cutaneous melanoma, malignant mesothelioma, renal cell carcinoma, meningioma, cholangiocarcinoma, BAP1-inactivated nevus. Non-melanoma skin cancer in case of unusually high frequency in a single individual or at unusually young age. In populations with a high incidence of cutaneous melanoma there should be ≥3 cutaneous melanoma if this is the only tumor type

*uveal melanoma <40 years, cutaneous melanoma <18 years, malignant mesothelioma <50 years, renal cell carcinoma <46 years.

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Chapter 3.2

The pediatric choroidal and ciliary body melanoma genetical study

A survey by the European Ophthalmic Oncology Group

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Submitted

ABSTRACT

Purpose
Our primary aim was to elucidate the genetic background among children and young adults with choroidal and ciliary body melanoma, with special focus on the *BAP1* germline variants contribution among this age group.

Design
Retrospective, multicentre observational study.

Participants
Ninety-three patients from thirteen ocular oncology centers were included, children were defined as younger than 18 years of age, while young adults aged 18 to 24 years. All patients had confirmed diagnosis of choroidal or ciliary body melanoma, records of clinical and histopathological data were used.

Methods
Data from medical records were available from a previous large collaborative study, data were filled using a secure website and were reviewed centrally. *BAP1* immunohistochemistry was used to evaluate the status of *BAP1* in the tumor. Next-generation sequencing using Ion Torrent platform (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine mutation status of the tumor or DNA extracted from blood or saliva of the following genes: *BAP1*, *EIF1AX*, *SF3B1*, *GNAQ* and *GNA11* and chromosome 3 status. Survival was analyzed using Kaplan-Meier survival estimates calculated with IBM SPSS Statistics (version 28.0.1.0).

Main outcome measures
Variables assessed include mutation status, chromosome 3 status, metastatic free survival, overall survival and gender.

Results
Of the Ninety-three patients 45 were children, and 39 were young adults. The mean age at diagnosis was 17.4 years (range 4.96-24.8). A *BAP1* germline pathogenic variant was identified in one patient (aged 18). The absence of a *BAP1* mutation and disomy 3 in the tumor tissue had the most favourable prognosis. Males showed better disease-free survival compared to females.

Conclusions
This study did not show *BAP1* germline predisposition among children and young adults with choroidal and ciliary body melanoma. Males had better survival. These findings confirm our previous results in the published survey of the Pediatric Choroidal and Ciliary Body Melanoma Study.

INTRODUCTION

Uveal melanoma (UM) is the most common primary intra-ocular malignancy among adults[1] with an estimated incidence of 7 per 1.000.000 people in the Western world.[2] The incidence varies from 2-8 cases per million in whites, depending on latitude.[3, 4] Congenital UM is relatively rare, with only few cases reported in the literature.[5-9] Pediatric UM (PUM), which affects pediatric and young adults age groups (<21 years) is extremely rare, and comprises less than 1-2% of all UM.[10-16]

PUM and adult UM harbor different clinical characteristics. Both melanomas are located primarily in the choroid, followed by iris and ciliary body,[11-13] but the incidence of iris melanoma among young adults (<21 years) is higher compared to adult UM.[15] Another difference is the smaller mean tumor diameter in young adults compared to elderly patients >60 years of age.[15] Higher percentage of female to male gender in PUM, was a strong evident, in a large collaborative study of 299 UM among children and young adults,[17]. This was in line with the prior meta-analysis by Al-Jamal *et al,*[10] whereas the prevalence of UM in adults is higher in males compared to females.[2]

Overall, PUM has a better prognosis compared to adult UM due to the lower metastatic rate.[11, 12, 15, 18] One study did not describe a better prognosis in children and young adults when adjusting for other factors.[16] Male children had a more favorable survival compared with females. However, this difference in prognosis was not observed in young adults (18-24 years of age).[17] Prognosis was not associated with tumor thickness or largest basal diameter (LBD) but the mortality was higher when the ciliary body was involved and when the TNM (tumor node metastasis) stage was higher.[10, 13, 17] Moreover, extraocular extension and the presence of congenital oculo(dermal) melanocytosis are poor prognostic factors.[17] One study reported that juvenile UM patients who underwent proton beam radiotherapy had a higher risk of metastasis, especially when there was a large retinal detachment six months post treatment.[13]

Genetic factors have been described to predict prognosis of UM patients as well. Loss of alleles at loci on chromosome 2 were already described in UM in 1986.[19] However, later research showed that loss of chromosome 3 is an important prognostic factor. Patients in which the UM showed monosomy 3 has a less favorable prognosis compared to UM with disomy 3.[20] Moreover, monosomy 3 if often accompanied by gain of chromosome 8q,[21, 22] and correlates with poor prognosis.[23, 24] Later research showed that the tumor suppression BRCA1-associated protein 1 (*BAP1*) gene, located on chromosome 3, plays an important role in prognostication of UM patients.[25] *BAP1* is a deubiquitinating enzyme (DUB) and plays an important role in the DNA repair mechanism.[26] Several

functions of *BAP1* are described as it is involved in cell regulation, metabolism and chromosome stability. [27-29] Moreover, it has been shown that loss of *BAP1* in uveal melanoma is correlated with increased transcriptome levels of CD38, HLA-DRA, IDO-1 and LAG-3 which are associated with immune suppressive pathways.[30] Despite this knowledge, the exact role of *BAP1* in the development of metastasis is yet unclear because of its many functions and interactions.

Patients with a *BAP1* mutated UM are more likely to develop metastasizing UM compared to UM without a *BAP1* mutation.[25] It have been shown that immunohistochemically nuclear loss of *BAP1* was associated with a *BAP1* mutation[31] and has a significant correlation on patient survival.[32] Therefore, immunohistochemistry of *BAP1* could be used to predict patients prognosis in UM.

Not only somatic mutations in *BAP1* are described in patients with UM, germline variants occur as well. The patients carrying a *BAP1* germline pathogenic variants also had other cancers besides UM, such as cutaneous melanoma, renal cell carcinoma, and mesothelioma.[33-35] This *BAP1* tumor predisposition syndrome is only present in a minority of all UM patients.[33] When looked to the age of onset of UM in patients with a null and missense variant, no significant difference was found.[34] However, these studies did not include younger age groups of UM patients, hence making the prevalence of germline variants in this specific group unknown.

Aims of the Study

In our published collaborative study "The Pediatric Choroidal and Ciliary Body Melanoma A Survey by the European Ophthalmic Oncology Group",[17] we found out that cytogenetic monosomy 3 was found in 54% of children (11 to 17 years) and in 24% of young adults (18-24 years). Also one patient in both groups tested positive for somatic *BAP1* mutations. Our study found preliminary evidence that the pathogenesis of PUM may differ in three age groups. Based on these observations, we aim to confirm or exclude that monosomy 3 and disomy 3 predicts higher and lower risk for metastasis respectively among PUM. The aim of this multicenter Ocular Oncology Group (OOG) study is to elucidate the genetic background of PUM, with a special focus on *BAP1* germline variants.

MATERIALS & METHODS

Patient selection

Eligible for this retrospective cohort study were all patients in which choroidal and ciliary body melanoma was diagnosed at an age younger than 25 years, and for whom at least the following data were available: birth date, date of diagnosis, gender, treatment type, presence or absence of local or systemic tumor recurrence, last survival status, date of last known status, and cause of death (UM, second cancer, or nonmalignant cause) determined by reviewing patient charts, registry data, histologic samples, and death certificates. Patients with iris melanomas were ineligible. All treatment methods were eligible. Informed consent of all patients was obtained before processing the samples. This investigation was approved by the institutional review boards of the participating centers as required and adhered to the tenets of the Declaration of Helsinki and was approved by the local ethics committees.

Data Collection

Data on consecutive eligible patients were collected from members of the European Ophthalmic Oncology Group (OOG). The data additionally acquired included presence of congenital oculo(dermal) melanocytosis or neurofibromatosis; visual acuity and intraocular pressure at diagnosis and at last visit; tumor thickness; largest basal diameter of tumor; ciliary body involvement; extraocular extension; tumor distance from the center of the fovea and the margin of the optic disc; tumor cell type; tumor cytogenetic features; dates of any local tumor recurrence; secondary enucleation, and metastasis; and second primary malignancies. We staged the tumors according to the seventh edition of the TNM system of the American Joint Committee on Cancer. [36] Participating ocular oncology services submitted data anonymously through a secure survey website from patients diagnosed between 1968 and 2018.

Blood was withdrawn or saliva was collected from patients and parents if possible. Germline testing was performed on retinal tissue in case no blood or saliva was available. Tumor material from enucleated eyes or biopsies were used for immunohistochemistry (IHC) and DNA isolation when available.

DNA extraction

Targeted NGS was performed on DNA extracted from blood, saliva, formalin fixed paraffin embedded tissue (FFPE) of retina and tumor. For DNA isolation of blood, the QIAmp DNA Blood kit was used (Qiagen, Hilden, Germany) according to the manufacturers protocol. DNA isolation from saliva was performed with the Oragene DNA OG-500 kit for collection of human DNA (DNA Genotek Inc., Ottawa, ON, Canada) following manufacturers protocol. FFPE sections were used to isolate normal tissue from the retina and tumor tissue. Depending on the

size of the tumor, 4-9 5µm FFPE sections were deparaffinized and hematoxylin stained prior to DNA isolation. DNA extraction was performed as described before with 5% Chelex (Bio-Rad, Hercules, CA, USA) and Proteinase K (Qiagen, Hilden, Germany).[37]
DNA was stored at -20C° and concentrations were measured using the Quant-iT™ dsDNA Assay Kit, high sensitivity (Thermo Fisher Scientific, Waltham, MA, USA) as described by the manufacturer.

Immunohistochemistry
BAP1 immunohistochemistry (IHC) was performed on FFPE tumor tissue. Sections of 4-5 µm were used for IHC and performed as described previously.[31] All slides were evaluated by an ophthalmic pathologist and one of the authors (RV and NP) for the presence of nuclear *BAP1* expression. Lack of nuclear *BAP1* expression was considered as a mutation in the *BAP1* gene.

Mutation analysis
A *BAP1* mutation was assumed in case of absent nuclear *BAP1* expression of tumor tissue. Subsequently, germline analysis for *BAP1* was performed on blood or normal FFPE tissue obtained from the ophthalmic slides. Next-generation sequencing (NGS) was performed using the Ion Torrent platform (Thermo Fisher Scientific, Waltham, MA, USA). A panel covering exon 4 and 5 of *GNAQ* and *GNA11*, exon 1 and 2 of *EIF1AX*, exon 14 of *SF3B1* and all exons of *BAP1* was used as described before.[37] The sequencing results were analyzed with Integrative Genomics Viewer (IGV) (Broad Institute, Cambridge, MA, USA). A mutation was considered when it occurs in a percentage of at least 10% of the reads with a minimal read count of 50.

Copy number variation
Copy number variation of chromosome 3 was performed with 21 amplicons covering highly polymorphic regions with a minor allele frequency of at least 45% as described previously.[37] Scatter plots were used to display the frequency of variant coverage compared to total coverage. This data was extracted from the variant calling files.

Statistical analysis
All analysis were performed with IBM SPPS Statistics Version 28.0.1.0 (IBM, Armonk, NY, USA). Kaplan-Meier estimates were used to compare survival between groups with the log-rank test accordingly. A P value < 0.05 was considered statistically significant.

Moreover, clinical, histopathological and genetic data was obtained of 28 pediatric UM patient from a participating institute.

RESULTS

Sample collection

A total of 93 patients were included, 67 patients were collected from twelve ocular oncology centers, and analyzed at the Erasmus Medical Center (Rotterdam, the Netherlands) while 26 samples were analyzed at the Curie Institute (Paris, France). The samples that were sent to the Erasmus Medical Center were either blood, FFPE, or saliva. Blood was collected from 18 patients, FFPE materials were avaliable for 41 patients and saliva was used for analysis from nine patients. From one patient blood as well as FFPE was used. A total of 68 samples from 67 patients were analyzed at the Erasmus Medical Center. A fine needle biopsy (FNB) was performed on three patients.

Clinical characteristics

This cohort consisted of 48 (55.8%) females and 38 (44.2%) males with a mean age at diagnosis of 17.4 years (range 4.96-24.8) (Table 1, Figure 1). Median age at diagnosis is only slightly higher with 17.6 years at diagnosis. The female male ratio was not significant different (*P*=0.332, binomial test). There was no significant difference in age at diagnosis between males and females (*P*=0.150, independent t-test). 70% of all patients were still alive at the moment of data collection, 15 patients died (16.1%). The status of 13 patients (14%) was unknown. From eight patients, the cause of death was metastatic disease from the primary UM. However, 12 patients (12.9%) developed metastatic disease. One patient died from another cause and from the rest the cause of death was unknown. Two patients underwent liver surgery for metastatic disease. One patient developed another primary malignancy, namely an intestinal adenocarcinoma.

Table 1. Clinical and histopathological characteristics of pediatric and young adult patients with uveal melanoma

Characteristic	n=
Sex	
Male	38 (44.2%)
Female	48 (55.8%)
Mean age at diagnosis (years)	17.4 (5.0-24.8)
Treatment	
Surgery	58 (62.3%)
Enucleation	50 (60.2%)
Local resection	6 (7.2%)
Endoresection	2 (2.4%)
Radiotherapy	41 (44.1%)
Ruthenium	12 (14.4%)

Table 1. Continued.

Characteristic	n=
Iodine	7 (8.4%)
Brachytherapy	3 (3.6%)
Proton beam	11 (13.2%)
External beam	3 (3.6%)
Cyber knife	3 (3.6%)
Gamma knife	2 (2.4%)
Metastatic disease	
Yes	12 (12.9%)
No	71 (76.3%)
Disease free survival (months)	**108.1 (0.1-524.5)**
Cell type	
Spindle cell	17 (50.0%)
Epithelioid	6 (17.6%)
Mixed	11 (32.8%)
Ciliary body involvement	
Yes	34 (41.0%)
No	49 (59.0%)
Extra ocular extension	
Yes	7 (8.5%)
No	75 (91.5%)
Mean largest basal tumor diameter (mm)	12.7 (4.0-20.4)
Mean maximum tumor thickness (mm)	8.1 (1.0-20.0)

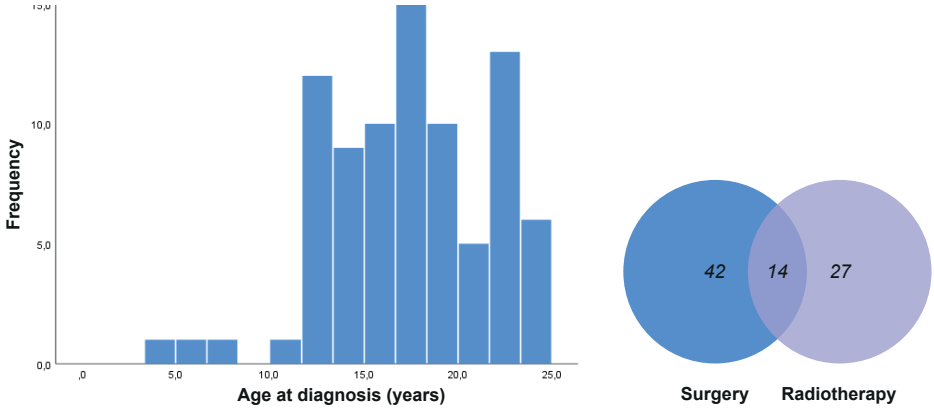

Figure 1. The histogram shows age at diagnosis (left), and the treatment of patients primary uveal melanoma is shown in the Venn diagram (right).

Mean disease-free survival (DFS) is 108 months (9.0 years) with a range of 0.1 months to 524.5 months. There was no significant difference in mean DFS between males and females (*P*=0.971, independent t-test). Overall survival (OS) was comparable to DFS with a mean of 9.1 years (range 0.01-43.7 years). However, the DFS between males and females was significantly different, males have a better prognosis compared to females (*P*=0.018, log-rank test, Figure 2A). However, no significant difference (*P*=0.058) was found between females and males regarding the presence of metastasis (Fisher's exact test). However, it should be noted that metastasis was present in 21.8% (10/46) in the female group whereas there was metastatic disease in 5.6% (2/36) in the male group. This did not meet statistical significance probably due to the small number of the patients and therefore limited power for this test. The presence of extraocular extension and ciliary body involvement did not reach significance between the different sexes (*P*=0.954 and *P*=0.705, Chi-Square). When looking at the age of diagnosis no overall difference between different age groups (0-17 years, 18-20 years, and 21-24 years) was found (Figure 2B). The pairwise correlation did not show any significant difference in DFS between the age groups. When we divided the age groups into younger and older than the age of 18 at diagnosis, no difference in DFS was observed either (*P*=0.562, log rank test). No difference was found in DFS in patients with or without extraocular extension or ciliary body involvement (Figure 2C,D). The DFS in patients with a *BAP1* mutation in the tumor (no *BAP1* expression using IHC or mutation detected with sequencing) was lower compared to patients without a mutation in *BAP1* (Figure 2E). Although small numbers, tumors with disomy 3 tend to develop less metastasis compared to tumors with loss of heterozygosity (*P*=0.027, pairwise comparison). However, log-rank testing of all groups did not show any significant difference (Figure 2F).

Histopathological features

From 34 UM the histopathological cell type was described. Most of these UM showed a spindle cell type (n=17, 50.0%). One-third consisted of mixed cell type (n=11) and six UM (17.6%) showed epithelioid cells. Ciliary body involvement was present in 34/83 (41.0%) and absent in 49/83 (59.0%). The presence of extraocular extension was described in 7 UM (8.5%), and no extraocular extension was present in 75 UM (91.5%) of the 82 UM of which the data was available.

The mean largest tumor diameter was 12.7mm (range 4.0-20.4mm) and the mean tumor thickness was 8.0 (range 1.0-20.0mm). TNM classification (TNM classification 7) was described in 56 cases. The most common TNM stage was T2a (n=15) followed by T3a (n=9) and T1a (n=7). The highest TNM classification was T4d, present in one patient.

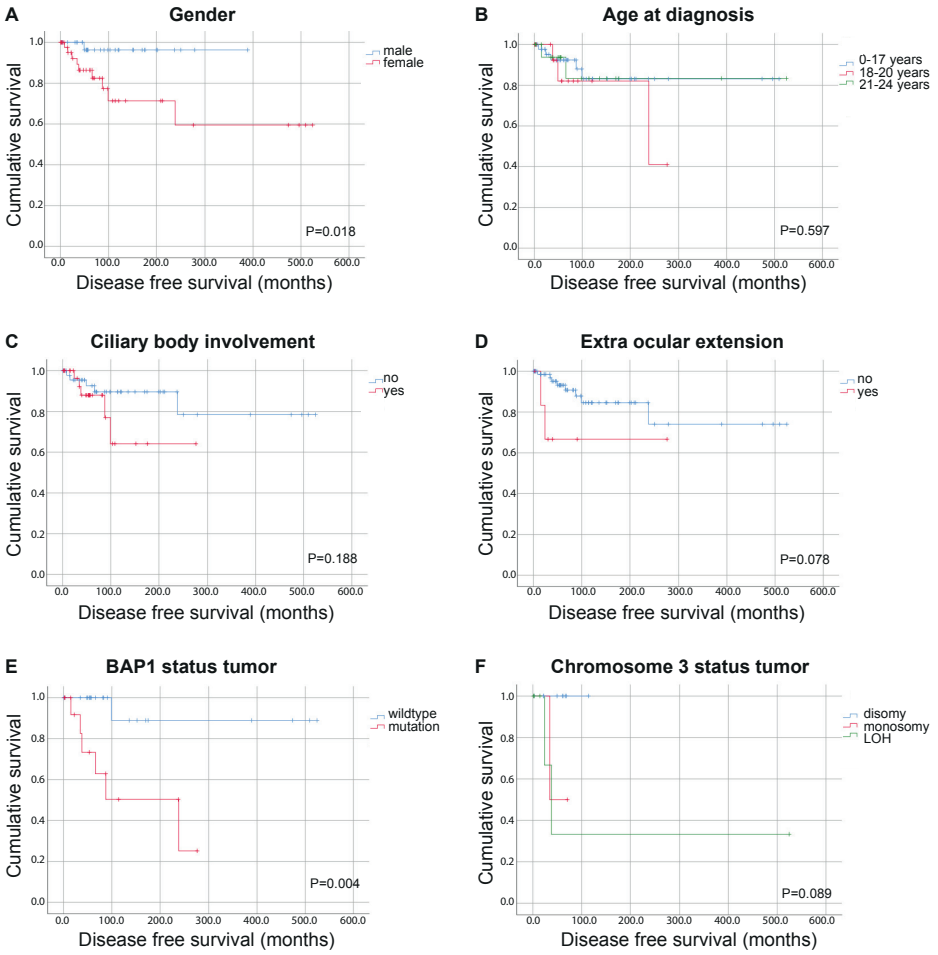

Figure 2. Kaplan-Meier estimates of patients with uveal melanoma. Disease free survival in months is displayed and compared between different groups according to **A)** gender, **B)** age at diagnosis, **C)** ciliary body involvement, **D)** extra ocular extension, **E)** *BAP 1* status of the tumor and **F)** chromosome 3 status of the tumor. LOH =loss of heterozygosity.

Treatment

Treatment consisted of surgery, radiotherapy, or a combination of both (Table 2, Figure 1). Of 10 patients (10.8%) no information on treatment was available. Surgery consisted of enucleation, endoresection, local resection, and in one patient iridocyclectomy was performed. Enucleation was performed in 50 patients (60.2%) of which ten patients received adjuvant treatment or enucleation was performed after primary treatment. Two patients were treated with endoresection followed by enucleation. Radiotherapy was performed in 41 patients (44.1%) of which ruthenium brachytherapy was used most followed by proton beam therapy. Fourteen patients (15.1%) were treated with a combination of surgery and radiotherapy. Resection and ruthenium brachytherapy (n=4),

enucleation and external beam radiotherapy (n=3), proton beam therapy and enucleation (n=2), endoresection and proton beam therapy (n=1), enucleation and cyber knife (n=1), enucleation and ruthenium (n=1), and enucleation and iodine (n=1). One patient was treated with local resection (iridocyclectomy), iodine radiotherapy, and enucleation.

BAP1 immunohistochemistry

BAP1 IHC was performed on 33 slides from UM. No nuclear staining was observed in 13 (39.4%) of these UM, considering the presence of a *BAP1* mutation. The other 20 UM showed *BAP1* expression (60.1%). Figure 3 shows UM with and without *BAP1* expression using IHC. Of all UM, a mutation of *BAP1* was considered in 14.0% using IHC.

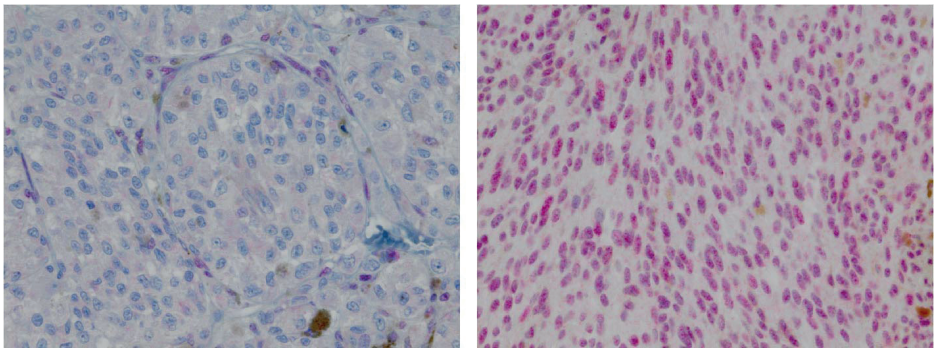

Figure 3. BAP1 immunohistochemistry of formalin fixed paraffin embedded tissue of uveal melanoma (400x). Absent nuclear *BAP1* expression (**left**), nuclear *BAP1* expression (**right**).

Next-generation sequencing

Next-generation sequencing of the tumor was performed on DNA isolated from FFPE of seven UM. *GNAQ*/*GNAQ11* mutations were detected in four UM and in one UM an *SF3B1* mutation was found (Table 2). No *EIF1AX* mutations were identified. *BAP1* mutations in the tumor were identified in three UM using NGS.

Reliable results of germline analysis of *BAP1* were obtained from sixteen blood samples, eight saliva samples, and eight FFPE samples. The prevalence of the *BAP1* germline variants was 3.1% (1/32, CI 95% 0.08 - 16.2) since one germline c.1708C>G, p.(Leu570Val) pathogenic variant was detected in a female patient. This variant results in a frameshift with a stop codon after 40 amino acids. The *BAP1* variant in this patient is described previously.[38] Age of onset was 18 years old and she underwent enucleation. No germline variants were detected in *GNAQ*, *GNA11*, *SF3B1,* or *EIF1AX* (Table 2).

Table 2. The findings of *GNAQ*, *GNA11*, *EIF1AX*, *SF3B1*, *BAP1* and chromosome status in the uveal melanoma tumor tissue and germline

	Tumor	**Germline**
GNAQ	c.626A>T, p.(Gln209Leu); c.548G>A, p. (Arg183Gln)	None
GNA11	c.626A>T, p.(Gln209Leu); c.626A>C, p.(Gln209Pro)	None
EIF1AX	None	None
SF3B1	c.1874G>A, p.(Arg625His)	None
BAP1	c.122+1G>A,; c.38-1G>C; c.442G>T, p.(Glu148*)	c.1708C>G, p.(Leu570fs*40)
Chromosome 3 Disomy	n= 9 (50.0%)	n= 32 (100%)
Chromosome 3 Loss of heterozygosity	n= 6 (33.3%)	n= 0 (0%)
Chromosome 3 Monosomy	n= 3 (16.7%)	n= 0 (0%)

Chromosome analysis of chromosome 3 was performed on 32 germline samples (blood, FFPE, or saliva) all resulting in disomy 3. Disomy 3 of the tumor was shown in 9/18 UM (50.0%), three UM (16.7%) showed loss of chromosome 3 (monosomy 3) and in six UM (33.3%) loss of heterozygosity (LOH) was observed.

DISCUSSION

In this collaborative study, we included 93 children and young adults with UM from thirteen centers. The mean and median age at diagnosis is at early adolescence namely at 17.4 and 17.6 years, respectively. The number of cases in this study is reasonable because this rare tumor is even more infrequent in children compared to adults.[15] UM in children and young adults differ from UM in adults in several characteristics. In adults, most UMs are located in the posterior choroid[15] whereas we observed ciliary body involvement in more than 40% of all patients. Also, other studies identified a larger percentage of iris and ciliary body UM in young patients compared to the adult population.[11, 15] Another difference is tumor diameter and survival of patients with UM in different age groups. The largest tumor diameter is higher in patients with UM > 60 years and children and young adults (<21 years of age at age of onset)[15] have a favorable prognsosis.[13, 15] Although the metastatic rate in juvenile patients is lower compared to adults, metastasis is still described in 19% of patients with an age at diagnosis of 20 years or younger, 15 years after diagnosis.[13] Metastasis at 10 years was 8.8% for children versus 25% for all ages, and metastasis at 20 years was 20.2% for children and 36% for all ages.[11] In this study it was shown that 12.9% of all patients developed the metastatic disease (12/93) which is

lower compared to previously mentioned studies. However, the follow-up time in our cohort is shorter with a mean DFS of 9 years and a median of 5.5 years. When looking at the metastatic rate in our study compared to metastasis at 10 years after diagnosis in other studies, our study showed only a slightly higher metastatic rate (12.9% vs 8.8%).[11]

In previous studies, it was shown that females are more affected than males[10], although this was not statistically significant in most studies.[11, 12, 17] In our study, more females were affected but this did not meet statistical significance. Probably the number of patients in each study is too low to statistically confirm this finding. We observed a higher DFS in male patients compared to females (*P*=0.018) indicating a more favorable prognosis in this group. This is in line with previously published studies in which gender was an independent predictor of survival after correction,[10, 17] of which one study showed that this was especially in the children with an age under seventeen.[10] Another predictor for survival in our study was chromosome 3 status and the presence of a *BAP1* mutation in the tumor. The prognosis was better when both copies of chromosome 3 were present in the UM. This is in line with the previous reported series in which monosomy 3 was correlated with poor survival. [20, 23] *BAP1* mutations in the tumor were observed in 41.5% of patients and these patients had a lower disease-free survival (*P*=0.004).

Not only do somatic *BAP1* mutations occur in UM, but also germline pathogenic variants in *BAP1* are described in UM. In adults, germline *BAP1* variants are present only in the minority of patients in unselected patient series.[39, 40] We did not observe a higher number of children with UM with germline *BAP1* pathogenic variants compared to adults. This is in line with the observation that the age of onset in adults is not lower for patients with an *BAP1* germline pathogenic variants compared to UM patients with a somatic mutation in the tumour.[40] When looking at the *BAP1* tumor predisposition syndrome, a hereditary tumor syndrome causing mesothelioma, cutaneous melanoma, renal cell carcinoma, and UM, there is a difference in age of onset regarding the type of *BAP1* variant. The age of onset was lower in patients harboring a null variant compared to a missense variant. However, this difference was not observed in UM and renal cell carcinoma.[34] This suggests that the pathophysiology of *BAP1* mutations in UM is different compared to other cancers. Moreover, this could support our results showing a low amount of germline variants in *BAP1* in children with UM, like in adults. The pathophysiology of the development of UM in children and young adults remains unclear and further research is necessary to elucidate underlying genetic predisposing factors.

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Chapter 4

Testing of uveal melanoma

Chapter 4

Combined mutation and copy-number variation detection by targeted next-generation sequencing in uveal melanoma

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ABSTRACT

Uveal melanoma is a highly aggressive cancer of the eye, in which nearly 50% of the patients die from metastasis. It is the most common type of primary eye cancer in adults. Chromosome and mutation status have been shown to correlate with the disease-free survival. Loss of chromosome 3 and inactivating mutations in *BAP1*, which is located on chromosome 3, are strongly associated with 'high-risk' tumors that metastasize early. Other genes often involved in uveal melanoma are SF3B1 and EIF1AX, which are found to be mutated in intermediate- and low-risk tumors, respectively. To obtain genetic information of all genes in one test, we developed a targeted sequencing method that can detect mutations in uveal melanoma genes and chromosomal anomalies in chromosome 1, 3, and 8. With as little as 10 ng DNA, we obtained enough coverage on all genes to detect mutations, such as substitutions, deletions, and insertions. These results were validated with Sanger sequencing in 28 samples. In 49.0% of the cases, the *BAP1* mutation status corresponded to the BAP1 immunohistochemistry. The results obtained in the Ion Torrent single-nucleotide polymorphism assay were confirmed with several other techniques, such as fluorescence in situ hybridization, multiplex ligation-dependent probe amplification, and Illumina SNP array. By validating our assay in 27 formalin-fixed paraffin-embedded and 43 fresh uveal melanomas, we show that mutations and chromosome status can reliably be obtained using targeted next-generation sequencing. Implementing this technique as a diagnostic pathology application for uveal melanoma will allow prediction of the patients' metastatic risk and potentially assess eligibility for new therapies.

INTRODUCTION

Uveal melanoma is the most common primary intraocular malignancy in adults with a worldwide annual incidence in Caucasians of 5–7 per million per year.[1] Despite successful treatment of the primary tumor, nearly 50% of the patients develop liver metastasis within 5 years. Once metastatic disease is diagnosed, survival is between 2 and 9 months.[2] Approximately 40% of uveal melanoma patients developed metastases within 4 years, but dissemination can occur even up to 4 decades after diagnosis.[3] This demonstrates that the prognosis for uveal melanoma patients can strongly vary between patients, and is dependent on a number of factors, including clinical and histological parameters, as well as the underlying genetic 'make up' of the tumor cells.[4] Chromosomal anomalies are often found in solid tumors, but previous work has shown that most of the chromosomal anomalies in uveal melanoma are limited to chromosome 1, 3, 6, and 8. Some of these chromosomal variations correlated with metastasis, such as loss of chromosome 3.[5] Monosomy 3 is observed in half of the patients and is strongly associated with poor survival. Loss of chromosome 3 is thought to be an early event, since it is present in the majority of the cells and often accompanies other chromosomal anomalies, such as gain of chromosome 8q.[6–8] Another common anomaly in metastasizing uveal melanoma with monosomy 3 is loss of chromosome 1p.[9] Chromosome 6 shows frequent rearrangements in both p- and q-arm in uveal melanoma; yet, deletion of 6q or gain of 6p are not associated with metastatic disease.[10] Uveal melanoma are genetically well-characterized tumors. Recent research using genome-wide sequencing led to the discovery of several genetic alterations, which correlate to a distinct survival pattern. Activating mutations in guanine-nucleotide-binding protein-Q (*GNAQ*) and -alpha 11 (*GNA11*) were found in the majority of uveal melanoma patients (83–93%), and are therefore thought to be initiating mutations.[11–13] Inactivating mutations in the BRCA-associated protein 1 (*BAP1*), located on chromosome 3p, were found in the early metastasizing patients.[14] Recently, two other genes have been reported that have a role in uveal melanoma biogenesis. Mutations in the eukaryotic translation initiation factor 1A (*EIF1AX*) were observed in non-metastasizing tumors15 and a hotspot mutation in the splicing factor 3 subunit 1 (*SF3B1*)-gene was detected in late metastasizing tumors.[16,17] Both of these genes are known to be mutually exclusive.

Current clinical diagnostics for uveal melanoma include several techniques, such as expression profiling,[18] copy-number analysis by Illumina single-nucleotide polymorphism (SNP)-array,[19] multiplex ligation-dependent probe amplification[20] or fluorescence in situ hybridization,[21] immunohistochemistry of the BAP1 protein,[22,23] and Sanger sequencing of *EIF1AX*, *SF3B1*, and *BAP1*. In some cases, whole genome sequencing or whole-exome sequencing is used to identify the somatic mutations present in the tumor.[15,24] In this study, we performed Ion

Torrent next-generation sequencing with a custom-made panel on 70 uveal melanomas to determine if targeted sequencing can be implemented in the routine uveal melanoma diagnostics. This panel has been designed specifically for uveal melanoma, covering all major hotspot mutations in the five relevant genes and several single-nucleotide polymorphisms on chromosome 1, 3, and 8 to allow analysis of clinically relevant chromosomal anomalies.

MATERIALS AND METHODS

Uveal Melanoma Samples

Sixty-five uveal melanoma samples were selected from our Rotterdam Ocular Melanoma Study Group database and 5 were external samples from patients who underwent enucleation, received for diagnostics from the Liverpool Ocular Oncology Research Group. Samples included in this study were diagnosed as uveal melanoma, collected between 1988 and 2016, and include formalin-fixed paraffin embedded and fresh specimens. A written informed consent was obtained before treatment, the study was performed according to the guidelines of the Declaration of Helsinki, and was approved by the local ethics committee.

DNA Extraction

Targeted next-generation sequencing was performed on DNA extracted from fresh- and formalin-fixed paraffin-embedded samples. For all tumor samples, an ophthalmic pathologist reviewed and selected tumor areas with an estimated minimal tumor cell percentage of 85%. DNA isolation from fresh tissue was carried out using the QIAmp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For formalin-fixed paraffin embedded samples, depending on the size of the tumor, 2–6 5 µm sections were de-paraffinized and haematoxylin-stained prior to isolation of the DNA. Formalin-fixed paraffin-embedded tumor tissue was micro-dissected by scraping the cells manually from haematoxylin-stained sections. DNA was then extracted by incubation of the tissues overnight at 56 °C in lysis buffer (Promega, Madison, WI, USA), containing 5% Chelex (Bio-Rad, Berkley, CA, USA) and Proteinase K (Qiagen). Proteinase K was inactivated by incubating the sample for 10 min at 95 °C and cell debris was pelleted down together with the Chelex by centrifugation in a micro centrifuge at maximum speed. DNA concentrations were measured with the Quant-iT Picogreen assay kit (Thermofisher Scientific, Grand Island, NY, USA), as described by the manufacturer. All DNA samples were stored at − 20 °C. The DNAs provided by the Liverpool Ocular Oncology Research Group had been extracted as previously described using the Qiagen DNeasy Blood and Tissue kit.[25]

Targeted Next-Generation Sequencing

A custom primer panel covering the five uveal melanoma genes and several SNPs located on chromosomes 1, 3, and 8, was designed using Ion Ampliseq Designer 2.0 (ThermoFisher Scientific). This resulted in an 11.5 kb amplicon panel, containing 98 amplicons. Amplicons designed for *GNAQ*, *GNA11*, *EIF1AX*, and *SF3B1* covered only the exons containing the known mutation hotspots. All exons of the *BAP1* gene were covered by amplicons. On chromosome 1 and 8, 17 amplicons were designed to cover highly polymorphic regions in the entire chromosome (Supplementary Table 1). These highly polymorphic regions with a global minor allele frequency of at least 45% were selected based on data found in the NCBI SNP database.[26] For chromosome 3, 21 amplicons were designed, due to the clinical relevance. The DNA input varied between 3 and 10 ng, depending on the amount of DNA available per sample. Library construction was performed using the AmpliSeq Library Kit 2.0. Next-Generation amplicon sequencing of the libraries was performed by semiconductor sequencing with the Ion Torrent Personal Genome Machine (Thermofisher Scientific) on an Ion Chip, according to the manufacturer's protocol.

Mutation Analysis

Adapter trimming and filtering of poor quality reads was performed on raw Ion Torrent sequence data by using the platform specific Torrent Suite Software V4.4.3 (Thermofisher Scientific). The generated sequence reads were analyzed with Coverage Analysis and Variant Caller v3.6 plugins to perform sequence coverage analysis and identify variants, respectively. Variants identified as a common polymorphism in the 1000 Genomes-database and variants that were present in 490% of the samples were excluded. If variants were present in a frequency higher than 15% and if they had a minimum read depth of 100 reads, they were called as mutations. Analysis of the detected mutations was done by visualizing the reads in Integrative Genomics Viewer software (Broad Institute, Cambridge, MA, USA) and comparing them to the Ensemble genome database (NM_002072; NM_002067; NM_004656; NM_012433; NM_001412).

Sanger Sequencing

DNA from 28 tumor samples was sequenced using the Sanger method to confirm results found by next generation sequencing. Selected regions of the genes of interest were amplified by PCR. Subsequently, sequencing of the PCR products and mutation analysis of *GNAQ*, *GNA11*, *BAP1*, and *SF3B1* and *EIF1AX* was done as reported previously.[13,16,22] Alignment of the sequence reads was done with reference sequence Hg19 from the Ensemble genome database.

Immunohistochemical Staining

To detect loss of the BAP1 protein in tumors, immunohistochemical staining of BAP1 was performed on 4 µm formalin-fixed paraffin-embedded sections of tumors. Staining was done by an automated immunohistochemistry staining system (Ventana Medical Systems, Tucson, AZ, USA) as described before.[22] BAP1 protein expression data were also available for the cases received from Liverpool Ocular Oncology Research Group, which were stained as previously described.[27] Sections were evaluated by the ophthalmic pathologists in Rotterdam and Liverpool (RV and SEC, respectively).

Copy-Number Variation Analysis

Validation of the copy-number status of the chromosomes was performed by SNP array, multiplex ligation-dependent probe amplification, and fluorescence in situ hybridization analysis. Two hundred nanograms of fresh tumor DNA was used for the Illuminia 610Q SNP array. Results were analyzed with Nexus Software (BioDiscovery, El Segundo, CA, USA). One hundred nanograms of DNA from each formalin-fixed paraffin-embedded uveal melanoma was used for multiplex ligation-dependent probe amplification analysis of chromosomes 1p, 3, 6 and 8 as previously described.[20] Fluorescence in situ hybridization analysis was performed on directly fixed tumor material, with probes for chromosome 1, 3, and 8 as reported previously.[21]

RESULTS

Coverage of uveal melanoma genes

To detect mutations in the *GNAQ*-, *GNA11*-, *EIF1AX*-, *SF3B1*-, and *BAP1* gene, 43 amplicons were used to sequence these genes reliably. Samples with a minimum total read count of 40.000 were analysed. for mutations in the five uveal melanoma genes. The total amount of read counts for fresh samples was on average slightly higher than those of formalin-fixed paraffin-embedded samples (Figure 1a). Most of the amplicons covering the five uveal melanoma genes consisted of 1–2% of the total read count, which corresponds to a minimum of 400 reads (Figure 1b). The median read count of all amplicons was 1.1%. Several amplicons obtained a coverage of <1% of the total read count, such as *EIF1AX* exon 1 and *BAP1* exon 1 and 3. By adding extra amplicons in the primer mix for these areas, we compensated for these lower read counts.

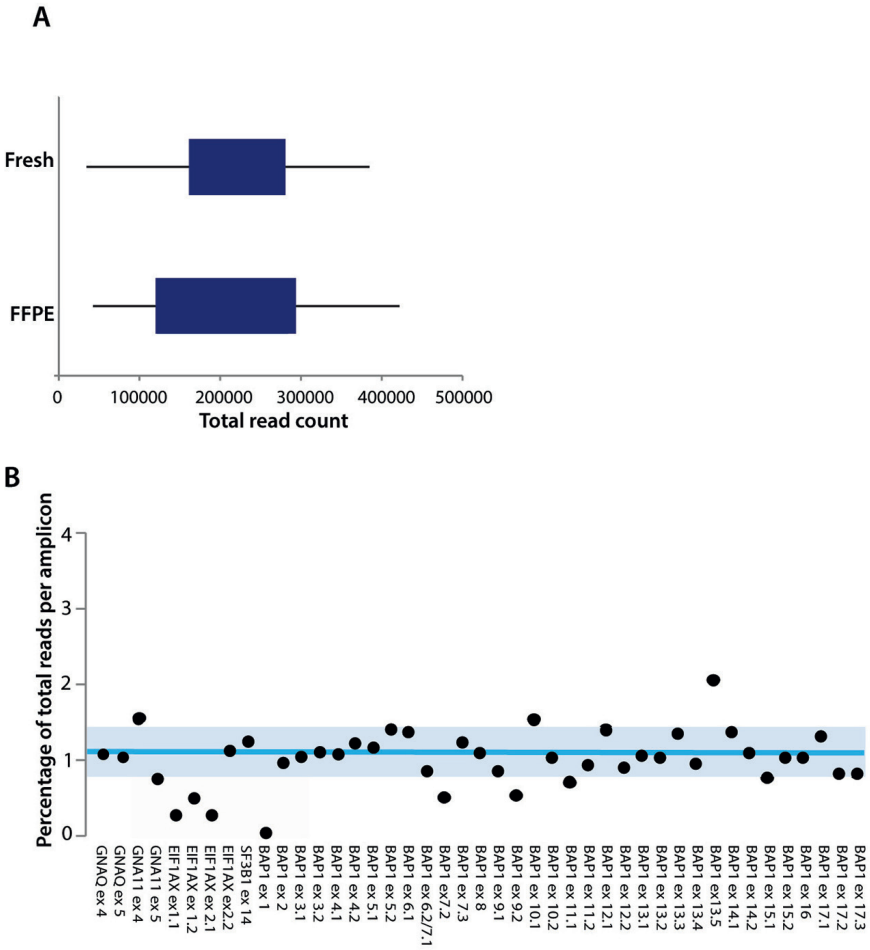

Figure 1. Sequencing efficiency of formalin-fixed, paraffin-embedded (FFPE), and fresh uveal melanoma specimens. **A)** Boxplots showing the total read count for all fresh- (**top plot**) and FFPE samples (**bottom plot**). **B)** Percentages of total reads visualized for all amplicons covering the five uveal melanoma genes. Solid line indicates median for all amplicons, and light gray area shows second- and third quartile.

Mutation Analysis

Seventy uveal melanoma samples were sequenced with our targeted panel. DNA was isolated from fresh specimens (n = 43) and from formalin-fixed paraffin embedded material (n = 27). From all 70 samples, sufficient DNA was extracted for sequencing. Forty-one percent of the samples harbored a *GNAQ* exon 5c.626A4C or c.626A4T mutation, 3% a *GNAQ* exon 4c.548G4A mutation, 41% a *GNA11* exon 5c.626A4T mutation, 1% a *GNA11* exon 4c.547C4T mutation, and in the remaining samples no mutations in either of these two genes were detected (Table 1). Mutations in the *BAP1* gene were found in 41% of the cases, mutations

in *SF3B1* in 16%, and *EIF1AX* in 20% of the samples (Supplementary Table 2). From 28 samples, we extracted enough DNA from fresh tissue to perform Sanger sequencing as well. All the mutations found by next-generation sequencing in these samples were validated by Sanger sequencing and no new mutations were identified.

Table 1. Amplicon location and mutation rate for the five genes relevant in uveal melanoma

Gene	Chromosome	Exons	Codons	Mutation rate (%)
GNA11	19	4, 5	183, 209	42
GNAQ	9	4, 5	183, 209	44
EIF1AX	X	1, 2	4-44	20
SF3B1	2	14	1873, 1874	16
BAP1	3	1-17	1-730	41

Detection of Loss of BAP1 Protein Expression

Absence of the BAP1 protein is often associated with monosomy 3 uveal melanoma. The loss of nuclear BAP1 expression can be immunohistochemically assessed, which is routinely performed in a diagnostic setting. Uveal melanoma samples were sequenced and analyzed for *BAP1* mutations. Half of all the samples showed loss of chromosome 3. Seventy-four percent of these monosomy 3 samples harbored a *BAP1* mutation and 26% did not. BAP1 immunohistochemistry was carried out for 59 samples, since we did not have tissue available for immunohistochemistry in all samples. In the BAP1- mutated samples of which we obtained BAP1 immunohistochemistry data, 80% showed a negative BAP1 immunohistochemistry (−), 5% showed a mixture of positive and negative BAP1 cells in the tumor (+/−), and 15% showed a positive BAP1 immunohistochemistry (+) (Figure 2 and Supplementary Table 2). The results obtained from three samples are depicted in Figure 3. Haematoxylin and eosin staining indicated a high presence of tumor cells in all three samples (Figure 3a). BAP1 staining was positive for the upper sample and negative for both the middle and lower samples (Figure 3b). Ion Torrent sequencing of the BAP1 gene revealed no mutations in the top sample but did show a mutation in the other two samples (Figure 3c), confirming the presence of BAP1 mutations in the immunohistochemistry BAP1-negative tumors.

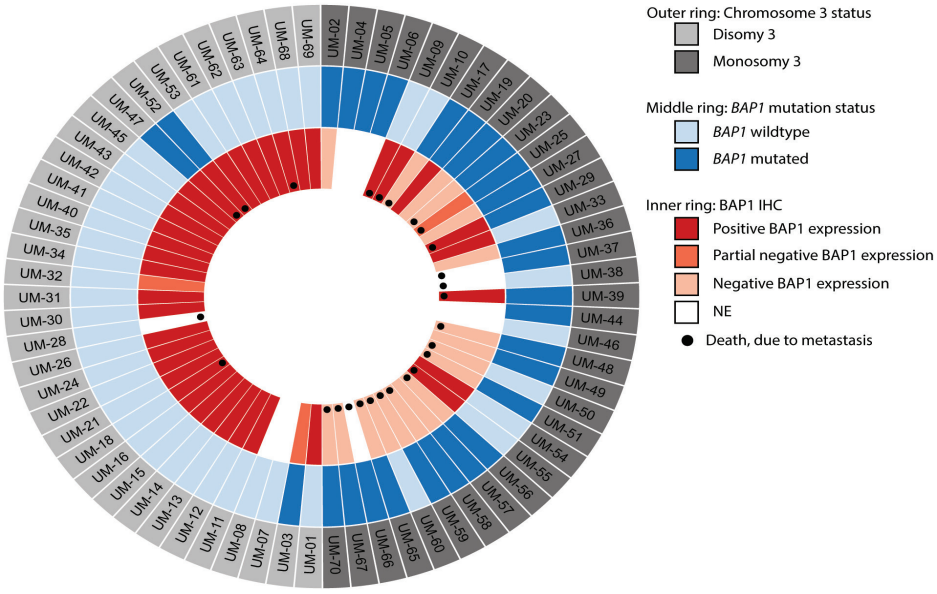

Figure 2. An overview of the overlap between the chromosome 3 status, *BAP1* mutation status and BAP1 expression. A doughnut chart visualizing the chromosome 3 status (outer ring), *BAP1* mutation status (middle ring), and BAP1 immunohistochemistry (IHC) (inner ring) for all 70 uveal melanoma samples.

Copy-Number Analysis SNP array, multiplex ligation-dependent probe amplification, and fluorescence in situ hybridization analyses are commonly used to identify chromosomal changes in tissues. To determine whether the Ion Torrent uveal melanoma custom panel allows a reliable detection of allelic imbalances caused by (partial) losses and gains of chromosome 1, 3 and 8, we compared results obtained by fluorescence in situ hybridization and SNP array with the copy-number variation results from our custom panel. Single nucleotide polymorphism covering amplicons were evenly distributed over the entire chromosome (Figure 4a), which allowed us to observe partial aberrations as well. Fluorescence in situ hybridization results showed disomy 3 for the top sample and monosomy 3 for the lower sample (Figure 4b). This was confirmed with the SNP array, where the log R Ratio and B-allele frequency shows no loss of heterozygosity for chromosome 3 in the upper sample and monosomy 3 for the lower sample (Figure 4c). The same pattern of allelic distribution was seen with the Ion Torrent single-nucleotide polymorphism analysis of chromosome 3 (Figure 4d). The B-allele frequencies for chromosome 1 and 8 were confirmed as well, as shown in Supplementary Figure 1. Across all samples, we found that 50% showed monosomy 3, 30% loss of chromosome 1p, and 57% gain of chromosome 8q. These percentages overlapped with the percentages found by other copy-number variation techniques. Thirty-four samples were validated with only an Illumina SNP array, 15 with SNP array, and fluorescence in situ hybridization, 7 with only fluorescence in situ hybridization, and 5 samples with multiplex ligation-dependent probe amplification (Supplementary Table 3).

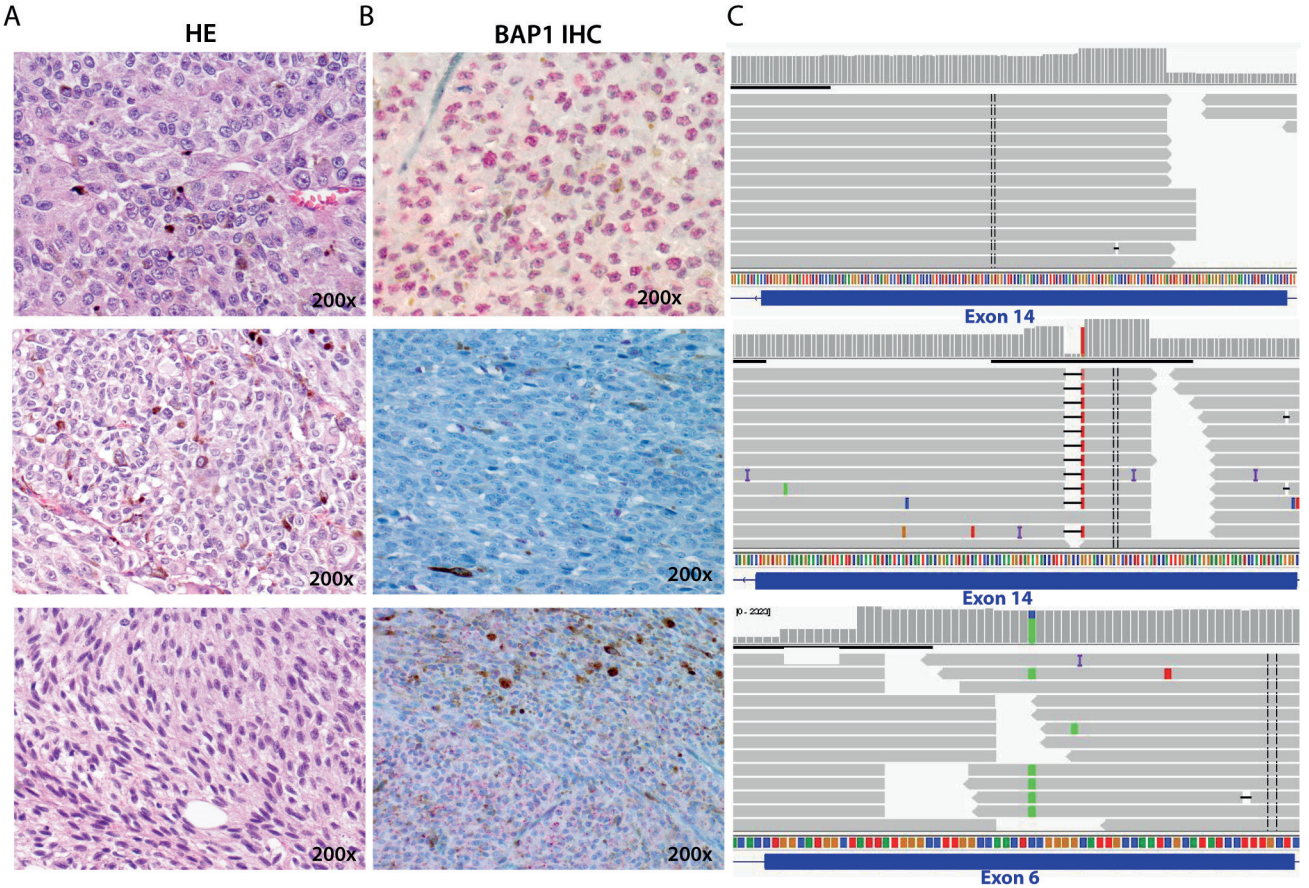

Figure 3. Histopathological and genetic aspects of three uveal melanoma specimens. **A)** Hematoxylin and eosin staining (HE) of three uveal melanoma samples (200x). **B)** Immunohistochemistry (IHC) staining of BAP1 protein showing strong nucleair BAP1 expression in the top sample and loss of BAP1 expression in middle en bottom sample (200x). **C)** From top to bottom: no mutation observed in the BAP1-gene, a 5 bp deletion and insertion in exon 4 resulting in a frameshift (c.175_179delinsA) and a point mutation in exon 6 that changes a glutamate into a STOP-codon (c.406G>T).

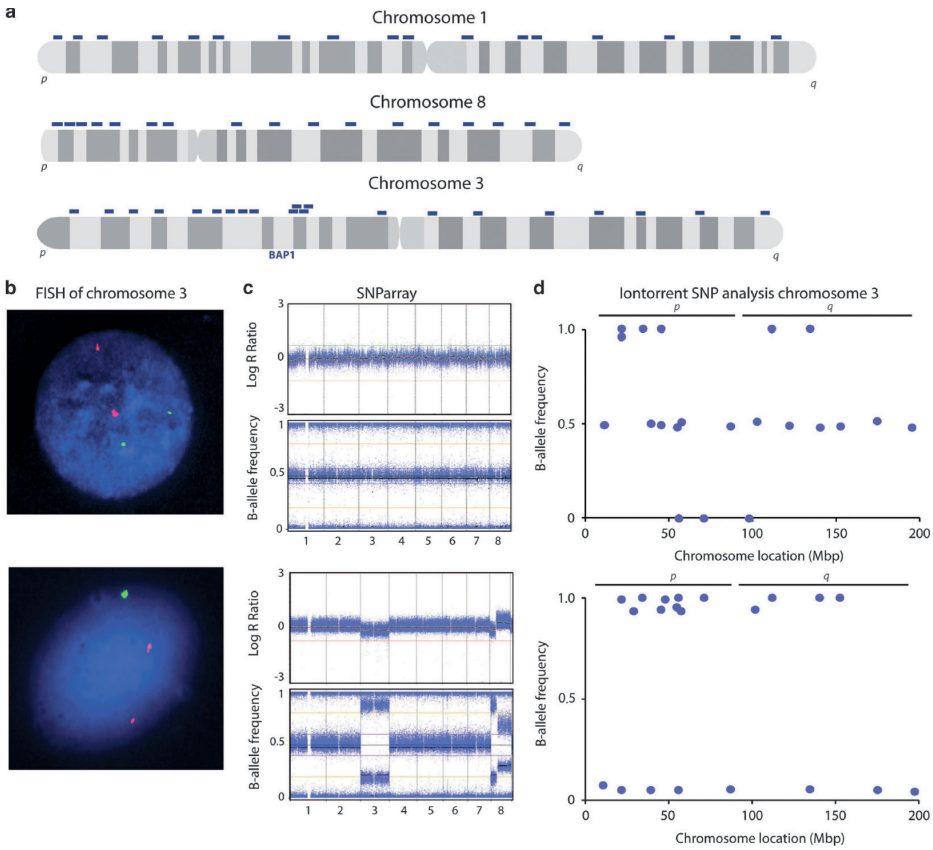

Figure 4. Copy-number analysis of chromosome 3. **A)** Visualization of the evenly spread amplicons covering highly polymorphic single-nucleotide polymorphisms on chromosome 1, 3, and 8. **B)** Fluorescence *in situ* hybridization (FISH) of chromosome 5 (red) and chromosome 3 (green) shows no loss for chromosome 3 in the top sample and loss of chromosome 3 in the bottom sample. **C)** Top SNP array visualizes chromosome status for chromosome 1–8. Both log R Ratio and B-allele frequency indicate disomy 3, whereas the SNP array for the bottom panel shows loss of chromosome 3. **D)** Single-nucleotide polymorphism (SNP) analysis performed by the targeted uveal melanoma panel visualizes the B-allele frequency for chromosome 3. Top single-nucleotide polymorphism analysis shows heterozygosity for the single-nucleotide polymorphisms, indicating disomy 3, while bottom sample shows no heterozygous variants indicating loss of heterozygosity of chromosome 3.

DISCUSSION

Uveal melanoma is characterized by recurrent mutated genes and chromosomal anomalies. In this study, we present a novel custom-designed next generation sequencing assay for uveal melanoma, which can be used to predict uveal melanoma patients' prognoses based on mutation status and chromosome status of chromosome 1, 3, and 8. The assay can be conducted with using either freshly isolated DNA or DNA obtained from formalin-fixed paraffin-

embedded material. This is the first study that establishes a method that can be used for uveal melanoma diagnostics on both formalin-fixed paraffin-embedded and fresh material. Our assay is cost-effective, since one method can replace techniques, such as fluorescence in situ hybridization, SNP array, and Sanger sequencing and it can be considered as a good alternative for BAP1 immunohistochemistry. Other important advantages are the low amount of DNA (10 ng) necessary for sequencing, which makes the technique suitable for transvitreal fine needle aspirations biopsies and the small amplicon size, allows sequencing of partially degraded DNA from formalin-fixed paraffin embedded tissue. Our assay could be performed on other next-generation sequencing platforms than Ion Torrent sequencing as well, if these two characteristics are taken into account. Furthermore, compared to other techniques that only identify the high-risk patients that metastasize early, this technique also allows us to identify the potentially late metastasizing patients that often harbor a *SF3B1* mutation.

Prognostication of uveal melanoma patients can be achieved by analyzing mutation status. Currently, this is usually performed by Sanger sequencing. Mutations in *GNAQ*, *GNA11*, and *SF3B1*, all gain of function mutations, occur almost exclusively in hotspot locations, therefore only these locations have to be sequenced. Since mutations can occur throughout the entire *BAP1* gene, large amounts of DNA are needed for the sequencing of multiple exons. Whole-exome sequencing is a reliable and easy method to obtain mutation status as well. However, since only a few genes are involved in the oncogenesis of uveal melanoma, many irrelevant reads will be produced. Whole-exome sequencing is less cost-effective for the diagnostic setting, compared to targeted Ion Torrent sequencing. Several regions of the human genome are difficult to cover with next-generation sequencing. As shown in Figure 1b, a few exons, such as *BAP1* exon 1 and the first two exons of *EIF1AX*, show a relatively low read count. Due to this low read count, it is more difficult to detect mutations in this particular exon. These findings are not only observed in our targeted uveal melanoma panel, but also in whole-genome sequencing data of uveal melanoma.[17,28] Since exon 1 of the *BAP1* gene is located in the non-translated region, the effect of a mutation in this UTR region is not always clear. Another region, which is sensitive for sequencing errors is exon 1 of *EIF1AX*, caused by a pseudogene on chromosome 1. Amplicons covering only exon 1 may also produce reads derived from chromosome 1. By adding a second set of reads generated by a different amplicon for *EIF1AX*, we now cover not only exon 1 but also a part of the 3'UTR, which will obtain longer reads that can only be derived from *EIF1AX* exon 1. In our cohort, we observed mutations in all of the major uveal melanoma genes. Eighty-six percent of the samples showed a mutation in *GNAQ* or *GNA11*. Mutations in *EIF1AX* were found in 20%, mutations in *SF3B1* in 16%, and mutations in BAP1 were detected in 41% of the cases.

The obtained results do not exactly overlap with the mutation rates for uveal melanoma that we previously reported,[16] but those differences can be explained by the bias in our sample population. Samples selected for this study were not randomly chosen, but rather selected based on follow-up length and tissue availability. Figure 2 shows that only 74% of the monosomy samples harbor a *BAP1* mutation, which can be explained by studies showing *that* BAP1 mutations arise after loss of chromosome 3.[29] Most of the *BAP1*-mutated samples showed a negative BAP1 immunohistochemistry, but some had positive and negative BAP1 immunohistochemistry cells, which possibly indicates that not all of the cells in the tumor have acquired the mutation yet. However, we also observed *BAP1*-mutated samples that showed a positive BAP1 immunohistochemistry. For the disomy 3 samples, this can be explained by the presence of a *BAP1* wild-type gene, but this is not the case for the monosomy 3 samples. In these samples, we hypothesize that the mutated mRNA is not degraded by nonsense mediated decay and could thereby still be translated into a partially functional or non-functional protein. If the antibody binds at a different location as where the mutation is found, it will show a positive immunohistochemistry. However, for the majority (91.6%) of the samples the uveal melanoma panel can correctly detect mutations corresponding to the observed loss of BAP1 expression.

Our Ion Torrent uveal melanoma panel is in the current state already suitable for implementation in uveal melanoma prognostication, with the advantage that it can easily be expanded by adding the more recently discovered genes into our panel. Recently, it has been reported that a small percentage of the uveal melanoma samples contain mutations in other spliceosome components, *SR2F2* and *U2AF1*. It is thought that these tumors act in the same way as *SF3B1*-mutated tumors.[32] Other rare alterations in uveal melanoma are mutations in *PCLB4* and *CYSLTR2*, which are downstream targets of *GNA11* and *GNAQ* and are thereby thought to be less suitable for prognostication.[33]

In summary, we present a next-generation sequencing-based assay that can readily be implemented as a diagnostic pathology application for uveal melanoma. Mutation and copy-number variation data can be obtained by one technique, which can reliably predict the patients' outcome and potentially assess eligibility for new therapies. At present, there is no successful treatment for metastasized uveal melanoma; however, with the development of new therapies, identification of high-risk patients will be very important, particularly in adjuvant therapy trials. Our custom-designed uveal melanoma panel will make a valuable contribution to the rapid stratification of uveal melanoma patients.

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Supplementary Table 1. List of the highly polymorphic single nucleotide polymorphisms (SNP) covered by the uveal melanoma panel

	SNP-number	Position (bp)
Chromosome 1	rs7418256	4,084,304
	rs7412149	9,579,964
	rs12048851	16,382,718
	rs10907287	18,497,478
	rs6425861	34,372,503
	rs639298	42,001,530
	rs11209106	68,001,206
	rs480304	82,123,485
	rs10493903	98,900,818
	rs17258467	120,323,058
	rs1752380	151,347,746
	rs3856201	163,736,341
	rs10753786	169,288,770
	rs2072040	175,096,333
	rs138685314	188,228,295
	rs6681013	215,154,797
	rs592197	234,817,283
Chromosome 3	rs1601368	10,829,535
	rs1549356	21,528,837
	rs7612272	28,816,226
	rs7648156	34,497,918
	rs1274960	39,192,542
	rs267218	45,633,834
	rs9311387	46,115,590
	rs295449	47,375,955
	rs3821659	54,987,923
	rs2702143	55,738,509
	rs9868630	56,012,096
	rs62259027	57,747,389
	rs9310190	70,420,837
	rs12497448	86,741,603
	rs1151334	102,257,506
	rs3749299	111,673,147
	rs4045771	121,962,478

Supplementary Table 1. Continued.

	SNP-number	Position (bp)
	rs975149	134,666,475
	rs1004009	152,754,481
	rs9866779	175,021,665
	rs11717776	197,569,559
Chromosome 8	rs2405488	2,141,263
	rs4498602	10,180,242
	rs17577614	15,470,729
	rs13275706	19,327,151
	rs6557699	23,602,610
	rs1882928	31,023,822
	rs10095600	36,911,156
	rs4147426	47,909,945
	rs10107875	60,526,565
	rs6995640	68,904,187
	rs2120410	79,844,006
	rs13261311	87,705,504
	rs4735258	94,935,937
	rs4734993	108,686,209
	rs2142250	117,093,062
	rs6415522	131,905,690
	rs7008457	145,536,593

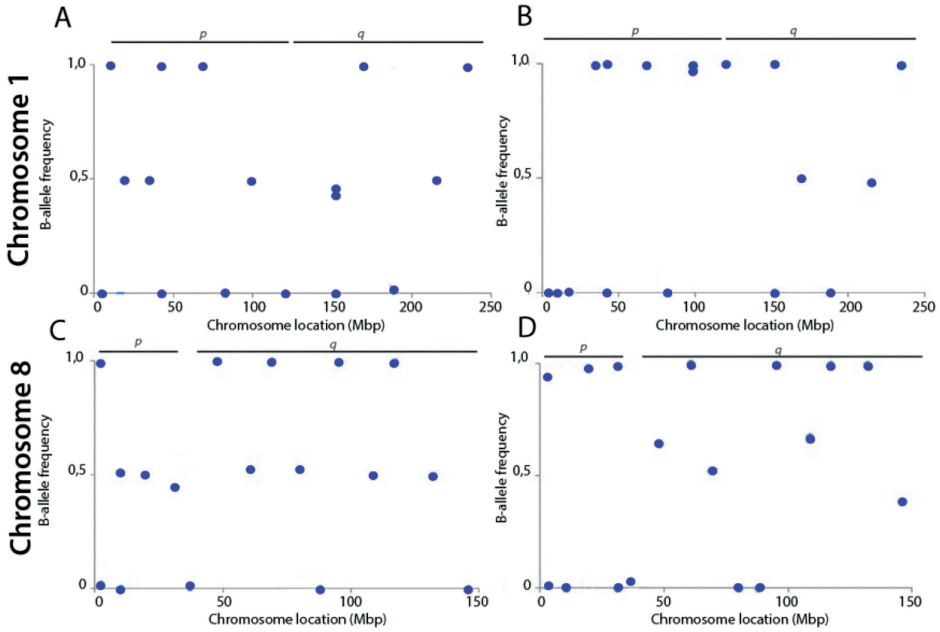
Supplementary Table 2. Mutation status, BAP1 immunohistochemistry and chromosome 3 status of all 70 samples

	Tissue	*GNAQ* ex 4	*GNAQ* ex 5	*GNA11* ex 4	*GNA11* ex 5	*EIF1AX* ex 1/2	*SF3B1* ex 14	BAP1	BAP1 IHC	Monosomy 3
UM-1	FFPE		•*			•*			+	
UM-2	FFPE		•					•	-	•
UM-3	FFPE		•					•	+/-	
UM-4	FFPE				•			•	ne	•
UM-5	FFPE		•					•	ne	•
UM-6	FFPE		•					•	ne	•
UM-7	FFPE				•		•		ne	
UM-8	FFPE		•			•			ne	
UM-9	FFPE								+	•
UM-10	FFPE				•				+	•
UM-11	FFPE				•		•		+	
UM-12	FFPE				•		•		+	
UM-13	FFPE				•		•		+	
UM-14	FFPE				•				+	
UM-15	FFPE				•				+	
UM-16	FFPE				•				+	
UM-17	FFPE				•			•	-	•
UM-18	FFPE		•			•			+	
UM-19	FFPE				•			•	+	•
UM-20	FFPE							•	-	•
UM-21	FFPE				•		•		+	
UM-22	FFPE				•		•		+	
UM-23	FFPE		•					•	-	•
UM-24	FFPE				•		•		+	
UM-25	FFPE				•*			•*	+/-	•
UM-26	FFPE		•*						+	
UM-27	FFPE				•*			•*	-	•
UM-28	Fresh								ne	
UM-29	Fresh		•*					•*	+	•
UM-30	Fresh					•			+	
UM-31	Fresh				•	•			+	
UM-32	Fresh								+/-	
UM-33	Fresh		•			•			+	•
UM-34	Fresh								+	
UM-35	Fresh				•*	•*			+	
UM-36	Fresh		•					•	-	•
UM-37	Fresh		•					•	ne	•

Supplementary Table 2. Continued.

	Tissue	*GNAQ* ex 4	*GNAQ* ex 5	*GNA11* ex 4	*GNA11* ex 5	*EIF1AX* ex 1/2	*SF3B1* ex 14	BAP1	BAP1 IHC	Monosomy 3
UM-38	Fresh				•*				ne	•
UM-39	Fresh				•*			•*	+	•
UM-40	Fresh		•*			•*			+	
UM-41	Fresh								+	
UM-42	Fresh				•*	•*			+	
UM-43	Fresh				•	•			+	
UM-44	Fresh		•*					•*	ne	•
UM-45	Fresh		•			•			+	
UM-46	Fresh				•				ne	•
UM-47	Fresh	•						•	+	
UM-48	Fresh			•*				•*	-	•
UM-49	Fresh	•						•	-	•
UM-50	Fresh		•*						-	•
UM-51	Fresh				•*			•*	-	•
UM-52	Fresh				•*			•*	+	
UM-53	Fresh		•*				•*		+	
UM-54	Fresh		•*			•*			+	•
UM-55	Fresh								+	•
UM-56	Fresh		•*					•*	-	•
UM-57	Fresh				•*			•*	-	•
UM-58	Fresh		•					•	-	•
UM-59	Fresh		•*					•*	-	•
UM-60	Fresh		•*						-	•
UM-61	Fresh		•*			•*			+	
UM-62	Fresh				•*		•*		+	
UM-63	Fresh		•*			•*			+	
UM-64	Fresh		•*				•*		+	
UM-65	Fresh		•*					•*	-	•
UM-66	Fresh		•					•	ne	•
UM-67	Fresh		•*					•*	-	•
UM-68	Fresh				•*		•*		+	
UM-69	Fresh								+	
UM-70	Fresh				•			•	-	

Mutation status
•: mutation observed; •*: mutation validated by sanger sequencing
BAP1 immunohistochemistry
+: positive BAP1 staining; +/-: mixed positive and negative BAP1 staining; -: negative BAP1 staining; ne: not evaluated
Copy number status
•: monosomy 3 observed



Supplementary Figure 1. Copy number analysis of chromosome 1 and 8. **A)** Single nucleotide polymorphism analysis indicates no loss of the entire chromosome 1. **B)** The absence of heterozygous variants in the B-allele frequency in the 1p arm of chromosome 1, indicates loss of 1p and normal 1q. **C)** Single nucleotide polymorphism analysis shows two copies of chromosome 8. **D)** Loss of the p-arm of chromosome 8 and ellelic imbalance of the 8q arm.

Supplementary Table 3. Chromosome status of chromosome 1p, 3 and 8q determined by Iontorrent single nucleotide polymorphism (SNP) assay or other copy number variation analysis techniques

	Loss of chromosome 1 p	Loss of chromosome 3	Gain of chromosome 8q
IonTorrent SNP assay	30% (19/63)	52% (33/63)	57% (36/63)
Other*	33% (20/61)	48% (29/61)	61% (37/61)

*SNP-array, multiplex ligation-dependent probe amplification and/or fluorescence in situ hybridisation analysis

Chapter 5

General discussion and summary

Chapter 5.1

General discussion

Melanoma of the conjunctiva, iris and posterior uvea behave differently regarding prognosis, metastatic sites and genetic background. With the studies described in this thesis I have set the first step to unravel the genetic changes that play a role in the development of these different types of ocular melanoma. Some of these have a strong hereditary component and investigations of the role of germline BAP1 mutations in children and adults are described. To conclude this thesis, the use of non-invasive testing in UM and future prospects are discussed.

SIMILARITIES AND DIFFERENCES OF OCULAR MELANOMA SUBTYPES

Uveal melanoma

In general, ocular melanoma is divided in uveal melanoma (iris, ciliary body and choroidal melanoma) and non-uveal melanoma (conjunctival melanoma) in clinical practice and research. Further sub classifications are made based on clinical behaviour and genetic differences. The prognosis of patients with iris melanoma, with a 10 years mortality of 5%,[1] is favourable compared to patients with posterior UM in which the 5-years mortality is about 40%.[2] When looking into the genetic background of iris melanoma, it is remarkable that mutations observed in iris melanoma are different from those in posterior UM since both tumours arise from melanocytes in the uveal tract. However, there are some overlapping genes which are involved in both iris and posterior melanoma such as *GNAQ*, *GNA11* and *EIF1AX*.[3,4] The more recently identified genes *CYSLTR2* and *PLCB4* that play a role in posterior UM are not yet extensively tested in iris melanoma. Scholz et al. included *CYSLTR2* in their next-generation sequencing panel for iris melanoma but no mutations were identified in contrast to the cohort of Johansson et al. in which two of the eight iris melanoma harboured a *CYSLTR2* mutation.[5] So far, no mutations in *PLCB4* were detected in iris melanoma thus additional testing of *PLCB4* and *CYSLTR2* in larger iris melanoma cohorts could be of interest.

Mutations in *GNAQ*, *GNA11*, *PLCB4* and *CYSLTR2* occur mainly in a mutually exclusive way in UM indicating that these four genes are involved in the same pathway.[6,7] Since cases are described in which more than one gene is affected,[5,7] it could be of benefit to test all UM for all these genes independent of their *GNAQ*/*GNA11* status. Johansson et al. showed that most cases in which *PLCB4* and *GNAQ*/*GNA11* co-occurred, residue R183 is affected instead of the more often affected residue Q209 in *GNAQ*/*GNA11*.[5] This suggests that the driver effect of different mutations is dependent on their position on the gene. So R183H mutations in *GNAQ*/*GNA11* might need an additional event, a *PLCB4* mutation for instance, to develop into a carcinogenic state.

An interesting finding is the recent identification of an Arg625Cys mutation in *SF3B1* in one iris melanoma case since this residue is affected in posterior UM as well.[4] No mutations in *SF3B1* were identified in a previous study,[3] possibly due to the low mutation rate of this gene in iris melanoma. However, in addition to our study in which two mutations in *SF3B1* were detected in iris melanoma, Johansson et al. also showed the presence of *SF3B1* mutations in iris melanoma.[5] The detection of *EIF1AX* mutations in iris melanoma and loss of BAP1 expression provide further support for an overlapping aetiology between iris and posterior melanoma.[3,4]

So there are some similarities between iris and posterior melanoma. Both are malignancies arising from the uveal tract and some of the genetic characteristics overlap. Remarkably, iris melanoma harbour mutations in a variety of genes that are also involved in other melanoma subtypes. Mutations in *BRAF*, *NRAS*, *PTEN*, *c-KIT* and *TP53* were detected in iris melanoma, mutations that are rarely involved in posterior UM.[4] Mutation in these genes are typically found in skin and conjunctival melanoma. This highlights the difference between iris melanoma and melanoma of the posterior segment. It could be suggested that the difference in structure or the location of the iris compared to the posterior segment might play a role. For example, a lighter iris colour and dark choroidal pigmentation was associated with posterior UM.[8] Moreover, an association between choroidal pigmentation and the number of choroidal melanocytes has been shown. So probably the composition of the different parts of the uveal tract could contribute to the development of UM since the amount of melanocytes differs between the anterior and posterior part. It seems unlikely that this difference is based already at the developmental status of the eye and that embryonic development contributes to differences in behaviour since the ciliary body (posterior UM) share the same embryonic origin as the iris.[9]

The role of ultraviolet light exposure could support the finding of overlapping gene mutations in cutaneous and iris melanoma. It could be suggested that the location of iris melanoma is correlated with sun-exposure since the lower hemisphere of the iris is more exposed to sunlight compared to the upper part and most iris melanoma occur in the inferior part of the iris.[1,10] In our study we did not detect an ultraviolet induced mutation signature and primary tumour locations were in different quadrants of the eye. When there would be a large effect of light damage one would expect that the tumours were mainly located in the lower half of the iris. The relation to ultraviolet damage is also not supported by studies in mucosal melanoma which harbour *SF3B1* mutations as in posterior UM, but also *BRAF*, *NRAS* and *c-KIT* mutations.[11] These melanoma have, like iris melanoma, genes involved which are known in posterior melanoma as well as cutaneous melanoma. Therefore, the role of ultraviolet damage based on the genetic profile of these melanoma subtypes can be argued since these

melanomas have no sun exposure. In contrast there are studies indicating that there is a higher tumour burden and features associated with UV-damage in iris melanoma. This was not seen in the more posteriorly located melanoma of the uveal tract.[5]

Regarding the location of *BRAF* mutations in iris melanoma and cutaneous melanoma, it is interesting that they share the same residues. In a small cohort of iris melanoma, V600 mutations were detected[12] which occur in cutaneous melanoma quite often.[13] A less common involved residue in skin melanoma, D594 which correlates with good prognosis[14] was affected in one iris melanoma.[4] This case is interesting since the tumour harbour a *GNA11* mutation as well, resulting in a genetic profile overlapping with both posterior UM and cutaneous UM.

Conjunctival melanoma

Conjunctival melanoma comprises only a small percentage of all ocular melanoma and has an incidence of 0.2-0.8 per million in the Western World.[15] It can arise from primary acquired melanosis (PAM), a nevus or de novo and can have a recurrent character.[16,17] Metastatic disease develop in less than one third of all patients, mostly to regional lymph nodes but distant metastases have also been described.[15,16] This is in contrast with uveal melanoma, which metastasizes haematogenous and mainly to the liver.[18] Extraocular extension is associated with poor survival as is incisional biopsy prior to treatment.[17] It has been shown that greater tumour thickness, a higher mitotic figure count, ulceration are also predictors of metastatic disease.[19] A correlation between metastasis free survival and the presence of a *TERT* mutations was evident in our study.[16] It should be noted that our cohort concerned relatively large lesions and also the location of the tumours could explain the inferior prognosis. The occurrence of *BRAF* mutations in the tumour did not correlate with prognosis which is congruent with previous studies in conjunctival melanoma.[20,21] The presence of *BRAF* mutations is interesting since this corresponds with the mutation signature of cutaneous melanoma in which *BRAF* mutations are frequently present at the same residue (V600).[17,22] Other mutations identified in conjunctival melanoma comprises mutations in *NRAS*, *PTEN* and *c-KIT*,[16,23] indicating that there are overlapping molecular features with cutaneous melanoma in which activators of the RAS-RAF-MEK-ERK pathway are also present.[24] In addition, the copy number variations found in conjunctival melanoma are more similar to cutaneous and mucosal melanoma than UM.[23] These findings suggest that the genetics of conjunctival melanoma are quite distinct from UM. Interestingly, mutations in *BAP1* with loss of BAP1 expression and even an *SF3B1* (p.Arg625His) mutation[16] were identified which are related to the genetic make-up of posterior UM.[25] However, to our knowledge no other conjunctival melanoma case harbouring an *SF3B1* mutation or *EIF1AX*[16] has been reported. In addition, no mutations in

GNAQ/*GNA11*[16,23] have been observed, suggesting a different molecular origin and tumour development of conjunctival melanoma compared to UM. It is interesting that an *SF3B1* mutation was found not co-existing with a *GNAQ*/*GNA11* mutation because these driver genes are mutated in UM and subsequently followed by mutations in *EIF1AX*, *SF3B1* and *BAP1*. This suggest that another driver gene is involved in conjunctival melanoma.

HEREDITY OF POSTERIOR UVEAL MELANOMA

UM is a rare disease and in the vast majority not of familial origin. However, UM families have been described and the underlying genetic predisposition is clear in some cases. Germline *BAP1* mutations are known to predispose for different types of cancer including UM. Most common tumours associated with the *BAP1*-tumour predisposition syndrome (BAP1-TPDS) are UM, mesothelioma, cutaneous melanoma, renal cell carcinoma, non-melanoma skin cancer, meningioma and cholangiocarcinoma.[26] The age of onset in patients with other tumours associated with the *BAP1*-TPDS is lower in tumours with a loss of function variant compared to a missense mutation except for UM and renal cell carcinoma. However, it should be noted that the age of onset was lower in all groups with germline *BAP1* mutations compared to the general population with these types of tumours and except for UM and renal cell carcinoma , the age of onset in patients with tumours associated with the *BAP1*-TPDS is lower in tumours with a loss of function variant compared to a missense mutation.[26] A possible explanation for the younger age of onset could be the role that *BAP1* plays in cell cycle regulation. It has been shown that *BAP1* has tumour suppression functions due to the involvement in cell death.[27] Since aging is accompanied by accumulative DNA damage,[28] it is possible that if tumour suppressor genes are affected, DNA damage occurs at a younger age. This might play a role in the younger age of onset of specific types of cancer in the *BAP1*-TPDS.

As the age of onset of *BAP1*-TPDS associated tumours is at a younger age it is interesting to study the role of germline *BAP1* mutations in children. Genomic instability and the accumulation of DNA damage are hallmarks of both aging and development of cancer which are intrinsic related to each other.[29] One could speculate on the genetic make-up of children that develop cancer that this might be prone to DNA damage since aging is not present. The paediatric uveal melanoma (PUM) study focussed on the *BAP1* gene, a gene which correlates with survival in UM and is known to play a role in familial cancer. In this study, children and young adults were included with uveal melanoma and *BAP1* testing was performed on tumour material. Germline testing for *BAP1* was performed when the UM harboured a *BAP1* mutation or in case no tumour material was

available. A germline *BAP1* mutation was only found in one patient (out of 93) which has been described previously as member of a large *BAP1*-TPDS family.[30] This suggest that germline *BAP1* mutations does not play a substantial role in the development of UM in children and young adults. However, we applied only targeted sequencing of the known UM genes so we cannot exclude the presence of germline mutations in other genes. A gene of interest is the p53 gene which can be mutated in cancer patients including children. A cohort of 268 children witch cancer showed a germline p53 mutation in 6.3% of cases (soft tissue sarcoma, osteosarcoma, brain tumour, adrenocortical carcinoma and neuroblastoma).[31] This percentage is clearly higher compared to *BAP1* germline mutations. However, in this study patients were selected based on the presence of cancer in combination with a positive family history for cancer, whereas the cohort of the PUM study was not adjusted for this criteria. It is a logical consequence that the amount of germline mutations is higher in a cohort in which family members are affected as well. If it is hypothesized that cancer at a young age occurs as a consequence of a genetic predisposition, the aim of future studies should be to unravel and identify sporadic genes that are involved as well as germline mutations. One strategy could be to perform whole exome or whole genome sequencing in order to identify genes that contribute to the development of cancer. UM specific genes could be identified to perform germline testing in patients in which familial aggregation of UM is present. When looking in a broader perspective in the light of cancer related syndromes such as the *BAP1*-TPDS, patients with a family history of cancer or a young age of onset should be tested. The first step in this process is to test only patients with UM and a first or second degree relative with a history of cancer to reduce the effects of noise in data acquisition since cancer is more common nowadays. There must be an indication of an underlying cause, so the rate of affected relatives should be higher than would be expected based on chance. Stricter criteria such as a young age of onset or more than one affected relative should be applied in case of cancers with a relatively high incidence. Testing in patients could be extended to a broader group when certain candidate genes are identified.

NON-INVASIVE TESTING AND PROGNOSTICATION MODELS

Clinical and histopathological parameters are used for prognostication of UM patients. Somatic mutations and chromosome aberrations correlate with patient prognosis, for example loss of chromosome 3 and *BAP1* mutated UM are known to be associated with poor survival. Several techniques have been described to evaluate the chromosomal status of the tumour in UM patients such as karyotyping, fluorescent in situ hybridization (FISH), single nucleotide polymorphism (SNP) array and next-generation sequencing.[32] These techniques

all require tumour tissue which is in general not available in patients who undergo eye persevering treatment. Exploring methods in which blood can be used to identify the chromosomal status of the primary UM is important since this can be used to predict a prognosis for patients. Both, circulating tumour DNA or cell free DNA (cfDNA) and circulating tumour cells (CTC's), has been used to predict prognosis of cancer patients and reflect the genetics of the primary tumour. It has been shown that ctDNA (circulating tumour DNA) can detect relapsing disease in breast cancer before this could be detected by conventional imaging.[33] This faces some difficulties in the light of UM since these are relatively small tumours with a significant lower tumour load compared to breast or prostate cancer.

Cell free DNA

For the usefulness of cfDNA as a biological marker in UM it is necessary to make use of a specific mutation to discriminate the tumour DNA from the normal DNA. Mutations in *BAP1* display a large variety from point mutations to large deletions which makes it difficult to use as a target, especially when the genetic profile of the primary tumour is not known. Probably *SF3B1* mutations, which are hotspot mutations, as well as *GNAQ*/*GNA11* mutations could be used for detection/analysis. The advantage of these hotspot mutations is that a specific target region can be analysed as these mutations have a high prevalence among UM. One of the drawbacks of this method is that the mutation profile of the metastases can differ from the primary tumour. This discrepancy between mutations is only rarely observed in UM whereas it is more common in other cancer types.[34] Harbour et al showed that UM harbour genetic alterations during their metastatic development and additional mutations occur during this process[7] but mutations from the primary tumour have also been detected in the metastatic tissue.[35] Thus, if a targeted digital PCR (dPCR) is developed for the detection of specific mutations in the blood of UM patients, this could be used as a diagnostic biomarker and probably as a tool for the detection of early metastatic disease when sensitive enough to detect these micro-metastasis. It has already been shown that ctDNA could be detected in patients with UM.[36] ctDNA levels were significantly higher in patients with metastatic UM compared to local disease, which makes this method suitable for the detection and follow-up of metastatic disease. Moreover, mutations in *GNAQ* and *GNA11* were detected in the ctDNA which demonstrates that this method is tumour specific.

CfDNA can also be used to detect genomic alterations in patients and healthy individuals. One of the methods is shallow sequencing, a spin-off of the non-invasive prenatal testing (NIPT) in pregnant women. Here blood of a UM patient will be used for screening for aneuploidy in the tumour. The genetic profile of UM is correlated with survival as described previously so the detection of loss of chromosome 3 in UM patients can be of prognostic value. This method could

be used at the time of diagnosis especially when eye preserving treatment is performed to gain some insight in the tumour characteristics based on genetic information. In a later stage this could be used as a follow-up method in patient who underwent surgery as well as patients treated with radiotherapy. We were able to detect copy number variations including loss of chromosome 3 in a patient with metastatic disease (unpublished data) using the NIPT test. The detection of copy number alterations in CTC's and cfDNA was also shown by Beasley et al. in metastatic UM.[36] If it is possible to detect copy number variations in patients with metastatic disease it could also be used at time at diagnosis. However, the tumour load is presumably lower at time at diagnosis compared to metastatic disease it is necessary to optimize this technique for UM patients first.

In patients who underwent eye persevering treatment the genetic profile of cfDNA and circulating tumour cells could be very useful to predict patients' prognosis. It has been shown that *GNAQ* and *GNA11* mutations can be detected in cfDNA of patients with metastatic or extraocular uveal melanoma using ultradeep sequencing.[37] Since *GNAQ* and *GNA11* have hotspot mutations in UM,[38] these genes are suitable for mutation analysis in cfDNA or ctDNA. *SF3B1* mutated UM are probably appropriate for this method as well because of their hotspot character whereas the detection of *BAP1* mutations in cfDNA will probably face many difficulties in the implementation of this method in daily practice. Many different mutations are described in *BAP1* as well as large deletions which makes it more difficult to detect these variants compared to hotspot mutations. However, a recent study shows the implementation of WGS in primary tumour material and blood samples in patients with metastatic disease.[39] Different tumour types were included showing that these methods are not tumour specific but can be used in a broader way. Both single nucleotide variants (SNV'S) as indels but also microsatellite instability and fusion genes were detected using this panel because of the use of tumour material and blood.

Despite the fact that the use of cfDNA might be more suitable for genes harbouring a hotspot mutation, it can be useful in different ways. When a systemic treatment for metastatic disease is developed these biomarkers could be used to evaluate therapeutic response. A big advantage relative to conventional imaging is the detection of micro-metastatic cells, which could not be detected on MRI or ultrasound. This will give more information in an early stage of the disease and probably lead to earlier treatment when a lower tumour burden is present. The correlation of ctDNA and prognosis is shown in patients with upper tract urothelial carcinoma as well as postoperative minimal residual disease.[40] This could be of clinical relevance in UM patients although no adjuvant treatment is available yet. Regarding future prospects when targeted treatment is available, this could be more specified to a patients' unique genetic

tumour profile. This could lead to an earlier and more effective treatment of metastatic disease.

Circulating tumour cells

The use of circulating tumour cells in UM has also been described in several studies. Anand et al. showed a correlation between the presence of CTCs and the development of metastasis in UM patients.[41] Moreover, CTCs were detected in patients with early stage UM and poor prognostic characteristics such as monosomy 3. Since CTCs were detected in almost one third of UM patients in this cohort, the absence of these cells in the blood could not be used as a predictor for a favourable prognosis yet. In patients who are at high risk for the development of metastatic disease, based on primary tumour characteristics, this method could be used to detect residual disease or the development of metastatic disease although the sensitivity is low.

Exosomes

Knowledge about the role of exosomes, nanosized extracellular vesicles present in body fluids, in cancer development has increased during the past decades. It has been shown that patients with metastatic UM have a higher amount of circulating exosomes in their blood compared to healthy controls.[42] mi-RNA profiles derived from exosomes demonstrated pathways involved in signalling and metabolisms which probably reflect the characteristics from the melanoma cells.[11] Moreover, a correlation between inflammation-related proteins in exosomes and metastatic disease in UM patients has been found.[43] This gives rise to the idea that different components of exosomes derived from blood of UM patients could be used in the prognostication of these patients. Since exosomes are present in many body fluids not only blood could be used for this purpose. The corpus vitreous which is anatomically close to posterior UM could be a target for biopsy, however a vitreous puncture which is performed with a needle through the sclera is not considered as a non-invasive technique. More approachable methods are the use of tears or anterior chamber fluid of which last mentioned is less invasive compared to the vitreous biopsy. All ocular fluids that could be used will face the same problem which is the limited amount of material that will be harvested for further research. The amount of fluid derived from the eye is only a fraction compared to blood or urine. This will make the isolation of exosomes more difficult especially when specific tumour derived exosomes are needed for further analysis. Besides these considerations it should be noted that the mi-RNA profile found in the vitreous is not necessarily the same as detected in exosomes isolated from the vitreous.[44]

All these genetic information from tumour tissue and when validated the non-invasive techniques could be used for prognostication of UM patients in clinical practice. One of the tools developed for this purpose is the Liverpool Uveal

Melanoma Prognosticator Online V3 (LUMPO3) which was validated in 2020.[45] In this model different covariates are included such as chromosome 3 and 8q status, tumour diameter, anterior margin, tumour thickness, extraocular extension, epithelioid cells, closed-loops and mitotic count.[46] It has also been shown that copy number variation profile correlated with mutation status. With the addition of clinicopathological data a multi-modality regression model was suitable for patients' survival prediction.[47] The use of these prognostication models could be relevant for clinical follow-up of patients. Surveillance protocols include blood tests for liver function and imaging such as ultrasound, MRI (magnetic resonance imaging) or CT (computed tomography). Drawbacks are the costs for the national health care system and in case of particular imaging the ionizing radiation. Screening frequency can be limited and therefore improve cost effectiveness and reduce risks such as radiation load with the implementation of risk calculation for metastatic disease.

To conclude, new approaches and methods in diagnostic and testing are promising for future prospects. The use of non-invasive testing techniques can be helpful in patients receiving eye preserving treatment in different types of ocular melanoma. Genetic characteristics of the primary tumour can be evaluated and used for prognostication and ctDNA levels can be used to detect the presence of minimal residual disease. Since the different types of ocular melanoma harbour their unique combination of genetic alternations and clinical characteristics it is possible to perform a more targeted diagnostic approach or use different genetic panels to make it more cost effective. When a non-invasive approach is added to prognosticate patients' prognosis, this would be a great step of improving patient care.

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Chapter 5.2

Summary

Ocular melanoma comprises an entity of melanoma of which all have their specific clinical, histopathlogical and genomic characteristics. In this thesis, several aspects of the different types of ocular melanoma are described.

In **chapter 1,** a general introduction on the different types of ocular melanoma (uveal melanoma and conjunctival melanoma) and their genetics are explained. Uveal melanoma (UM) is the most common form of ocular melanoma and can be divided into anterior (iris) and posterior (ciliary body and choroidal melanoma). Iris melanoma is less common and has a favorable prognosis compared to posterior melanoma. The genetic profile of posterior melanoma is correlated with survival, patients with posterior UM and monosomy 3 or loss of BAP1 expression in their tumour are most likely to develop metastatic disease, mainly to the liver. Metastases in patients with an *SF3B1* mutated tumour can develop even after 10 years from identification of the primary tumour.
Metastatic disease in conjunctival melanoma is detected mainly in lymphnodes and correlated with another genetic profile compared to UM. *TERT* and *BRAF* mutations, occuring in cutaneous melanoma as well, play a role in de development of conjunctival melanoma.
Not only somatic mutations occur in UM since families with an underlying germline mutation in *BAP1* are described. In these families with the *BAP1* tumour predisposition syndrome, several forms of cancer occur including UM. Germline testing in families with a high prevalence of specific tumours can be of additional value.

Chapter 2 comprises several paragraphs concerning different types of ocular melanoma and genetic backgrounds of these tumour types. In **chapter 2.1** iris melanoma and iris nevi are described in more detail. Not only mutations that occur in posterior UM were detected, but mutations that are found in cutaneous melanoma were identified with next-generation sequencing. One *SF3B1* mutation (Arg625Cys) was detected and mutations in *NRAS*, *BRAF*, *PTEN*, *c-KIT* and *TP53* were present. No significant correlation between BAP1 expression and survival was found. Interestingly, mutations in iris nevi were detected in *GNAQ*, *GNA11* and *BAP1* without loss of expression of BAP1. Since these borderline malignant nevi harbour potential malignant characteristics these nevi could be considered as iris melanocytic tumours of uncertain malignant potential.
The genetic background of conjunctival melanoma is highlighted in **chapter 2.2**. These melanoma harbour characteristics of posterior UM and cutaneous melanoma in which *BRAF* is frequently mutated. A correlation between *TERT* promotor mutation status and survival was identified. Patients with a conjunctival melanoma harbouring a mutation in this gene were more likely to develop metastatic disease compared to patients with a *TERT* wildtype melanoma. A mutation in *SF3B1*, the same residue as affected in UM, was detected. To our knowledge this is the first case described with this mutation.

An interesting phenomenon called chromothripsis is described in **chapter 2.3**. Parts of chromosomes are rearranged and this was found in almost 3% of our ROMS cohort of UM. Different chromosomes were affected by chromotripsis as well as specific co-concurrent driver mutations occurring in UM. In one tumour chromothripsis in two chromosomes was identified. Since chromothripsis is rare, further statistical analysis regarding survival was precluded.
The focus of **chapter 2.4** are spliceosome genes that were interesting to analyse in UM. Mutations in *SF3B1*, a gene involved in splicing, is not only affected in UM but occurs in myelodysplastic syndrome (MDS) as well. Tumours with an *SF3B1*-like chromosomal profile were selected and tested for mutations in *SRSF2* and *U2AF1*, genes involved in splicing and MDS. *SRSF2* mutations were detected in 2 UM (out of 42), all being in-frame deletions, whereas the mutations in MDS are missense mutations.

Chapter 3 outlines the heredity of UM. A general overview of germline mutations in *BAP1* is described in **chapter 3.1.** Germline mutations in *BAP1* are described in the *BAP1* tumour predisposition syndrome (*BAP1*-TPDS) underlying different types of cancer such as UM, malignant mesothelioma, renal cell carcinoma and basal cell carcinoma. Family history is therefore important in clinical practice and guidelines for germline screening are proposed. These guidelines included not only specific cancer types occurring in the family but also age of onset.
In **chapter 3.2** an international collaborative study from the Ocular Oncology Group (OOG) is presented. In this study children and young adults with UM were tested for germline mutations in *BAP1*. Moreover, clinical data was collected and it was found that boys have a favorable prognosis compared to girls. Metastatic disease was present in 13% of the 93 patients included in this study. Only one germline mutation in *BAP1* was detected which was previously described. This suggests that germline mutations in *BAP1* are not the predisposing factor of development of UM in children and young adults.

Chapter 4 describes a technique to identify mutations in UM using next-generation sequencing (NGS). A panel was developed and tested in fresh and formalin-fixed paraffin-embedded (FFPE) tumour material derived from UM. The genes included in this panel were *GNAQ*, *GNA11*, *BAP1*, *SF3B1* and *EIF1AX*. Moreover, SNPs covering highly polymorphic regions of chromosome 1, 3 and 8 were included to perform copy number variation analysis. This technique was validated using Sanger sequencing and SNP-array analysis. Moreover, BAP1 immunohistochemistry was used for *BAP1* analysis. This NGS technique is an alternative for Sanger sequencing and an advantage is the possibility to use DNA obtained from FFPE material which is often fragmented and therefore facing difficulties in other types of sequencing.

The main findings of this thesis are discussed in **chapter 5**. The differences and similarities of the individual types of ocular melanoma are elucidated and the role of germline mutations in UM is discussed. Finally, the role and possibilities of non-invasive testing and future prospects are described.

Chapter 5.3

Samenvatting

Oculair melanoom is de verzamelnaam voor een aantal verschillende vormen van oogmelanoom welke elk hun eigen specifieke klinische, histopathologische en genomische eigenschappen hebben. In dit proefschrift worden verschillende aspecten van de afzonderlijke vormen van het oculaire melanoom beschreven.

Hoofdstuk 1 geeft een algemene introductie over het oculaire melanoom. De verschillende vormen (uveamelanoom en conjunctiva melanoom) en hun genetische achtergrond worden uitgelegd. Het uveamelanoom (UM) is de meest voorkomende vorm van oculair melanoom en wordt verdeeld in anterieur (iris) en posterieur (corpus cilaire en choroideamelanoom). Het iris melanoom is minder voorkomend en heeft een betere prognose ten opzichte van het posterieure melanoom. Het genetische profiel van de posterieure melanomen is gecorreleerd met overleving, patiënten met posterior UM en monosomie 3 of verlies van BAP1 expressie in hun tumor hebben de hoogste kans op het ontwikkelen van metastasen, voornamelijk in de lever. Metastasen in patiënten met een *SF3B1* gemuteerde tumor kunnen zelfs 10 jaar na het vaststellen van de primaire tumor nog ontwikkelen.
Gemetastaseerde ziekte van conjunctiva melanomen wordt meestal als eerst vastgesteld in drainerende lymfklieren en zijn gecorreleerd met een ander genetisch profiel in vergelijking met UM. *TERT* en *BRAF* mutaties, die voorkomen in cutane melanomen, spelen een rol in de ontwikkeling van conjunctiva melanomen.
Niet alleen somatische mutaties komen voor in UM aangezien er ook families beschreven zijn met een onderliggende kiembaan mutatie in *BAP1*. In deze families met het *BAP1* tumor predispositie syndroom komen verschillende vormen van kanker voor, waaronder het uveamelanoom. Het kan daarom zinvol zijn om te testen of er kiembaanmutaties aanwezig zijn in families met een hoge prevalentie van specifieke tumoren.

Hoofdstuk 2 omvat een beschrijving van verschillende typen van het oculaire melanoom en de genetische achtergrond van deze tumoren. In **hoofdstuk 2.1** worden iris melanomen en iris nevi in meer detail beschreven. Niet alleen mutaties die voorkomen in posterieure UM werden gedetecteerd, maar ook mutaties die gevonden worden in huidmelanomen werden vastgesteld middels next-generation sequencing. Een *SF3B1* mutatie (Arg625Cys) werd gevonden en daarnaast waren mutaties in *NRAS*, *BRAF*, *PTEN*, *c-KIT* en *TP53* aanwezig. Er werd geen significante correlatie tussen BAP1 expressie en overleving gevonden. Interessant genoeg werden er mutaties gedetecteerd in *GNAQ*, *GNA11* en *BAP1* zonder verlies van BAP1 expressie in iris nevi. Aangezien deze borderline malignant nevi potentieel maligne kenmerken bevatten zouden deze als iris melanocytaire tumoren met onzeker maligne potentieel beschouwd kunnen worden

The genetische achtergrond van conjunctiva melanomen wordt toegelicht in **hoofdstuk 2.2**. Deze melanomen bevatten eigenschappen van zowel posterieure UM als huidmelanomen en bevatten vaak *BRAF* mutaties. Een correlatie tussen *TERT* promotor status en overleving werd vastgesteld. De conjunctiva melanoom patiënten met een mutatie in dit gen ontwikkelden vaker metastasen vergeleken met patiënten met een *TERT* wildtype melanoom. Een mutatie in *SF3B1*, hetzelfde aangedane residu als bij UM, werd gevonden. Voor zover bekend is dit de eerste casus waarbij deze mutatie beschreven wordt.
Een interessant fenomeen genaamd chromothripsis is beschreven in **hoofdstuk 2.3**. Door een onbekende oorzaak valt een chromosoom uiteen en deze stukken chromosoom worden opnieuw gerangschikt. Dit werd in bijna 3% van ons ROMS UM cohort gevonden. Verschillende chromosomen waren aangedaan evenals verschillende primaire en secundaire driver mutaties die voorkomen in UM. In één tumor was chromothripsis aanwezig in twee chromosomen. Omdat chromothripsis zeldzaam is kon er geen verdere statistische analyse betreffende overleving verricht worden.
De focus van **hoofdstuk 2.4** ligt op andere genen die interessant waren te analyseren in UM. Mutaties in *SF3B1*, een gen wat betrokken is bij *splicing*, is niet alleen aangedaan in UM maar komt ook voor bij met myelodysplastische syndroom (MDS). Tumoren met een *SF3B1* geassocieerd chromosoom profiel werden geselecteerd en getest op mutaties in *SRSF2* en *U2AF1*, genen betrokken in *splicing* en MDS. *SRSF2* mutaties werden in 2 UM (van de 42) gevonden, allen *in-frame* deleties terwijl *missense* mutaties in MDS gevonden worden.

Hoofdstuk 3 beschrijft de erfelijkheid van UM. Een overzicht van kiembaan mutaties in *BAP1* is beschreven in **hoofdstuk 3.1**. Kiembaan mutaties in *BAP1* zijn beschreven in het *BAP1* tumor predispositie syndroom (*BAP1*-TPDS), onderliggend aan verschillende typen kanker zoals UM, maligne mesothelioom, renaal cel carcinoom en basaal cel carcinoom. Een familieanamnese is daarom belangrijk in de praktijk, en een voorstel voor richtlijnen voor het screenen van kiembaan mutaties werd gedaan. In deze richtlijn worden de soorten kanker die voorkomen in de familie meegenomen maar ook de leeftijd waarbij kanker ontstaat.
In **hoofdstuk 3.2** wordt een internationale studie van de Ocular Oncology Group (OOG) beschreven. In deze studie worden kinderen en jongvolwassenen met UM getest op kiembaan mutaties in *BAP1*. Daarnaast werden klinische gegevens verzameld en werd gevonden dat jongens een betere prognose hadden ten opzichte van meisjes. Gemetastaseerde ziekte was aanwezig in 13% van de 93 patiënten die werden geïncludeerd in deze studie. Enkel één kiembaan mutatie in *BAP1* werd vastgesteld welke al eerder beschreven is. Dit suggereert dat kiembaan mutaties in *BAP1* niet de predisponerende factor zijn in de ontwikkeling van UM in kinderen en jongvolwassenen.

Hoofdstuk 4 beschrijft een techniek om mutaties in UM te identificeren met de hulp van next-generation sequencing (NGS). Een panel werd ontworpen en getest in vers en *formalin-fixed paraffin-embedded* (FFPE) tumor materiaal afkomstig van UM. De genen die in dit panel geïncludeerd werden zijn *GNAQ*, *GNA11*, *BAP1*, *SF3B1* en *EIF1AX*. Daarnaast werden SNPs op chromosoom 1, 3 en 8, die gebieden dekken die hoog polymorf zijn, geïncludeerd om *copy nummer variatie* analyses uit te kunnen voeren. Deze techniek werd gevalideerd door het gebruik van Sanger sequencing en SNP-array analyse. Daarnaast werd BAP1 immunohistochemie gebruikt voor de analyse van *BAP1*.
Deze NGS-techniek is een alternatief voor Sanger sequencing en heeft als voordeel dat het mogelijk is DNA verkregen uit FFPE te gebruiken. Dit DNA is vaak gefragmenteerd is en levert daarom vaak problemen op bij andere vormen van sequencing.

De voornaamste bevindingen van dit proefschrift worden bediscussieerd in **hoofdstuk 5**. Verschillen en overeenkomsten van de afzonderlijke typen oculair melanoom worden opgehelderd en de rol van kiembaan mutaties in UM besproken. Tot slot wordt de rol en mogelijkheden van niet-invasieve testen en toekomstperspectieven beschreven.

Chapter 6

Epilogue

Chapter 6.1

List of abbreviations

AJCC	American Joint Committee on Cancer
BAP1	BRCA1-associated protein 1
BAP1-TPDS	*BAP1* tumour predisposition syndrome
bp	base pair
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA1	breast cancer 1
cDNA	copy deoxyribonucleic acid
cfDNA	cell-free DNA
CNV	copy number variation
CM	conjunctival melanoma
CT	computed tomography
CTC	circulating tumor cell
ctDNA	circulating tumor DNA
CYSLTR2	cysteinyl leukotriene receptor 2
DFS	disease-free survival
DNA	deoxyribonucleic acid
EIF1AX	eukaryotic translation initiation factor 1A,X-linked
FFPE	formalin-fixed, paraffin-embedded
FISH	fluorescence in situ hybridization
GNAQ	guanine nucleotide-binding protein G subunit alpha Q
GNA11	guanine nucleotide-binding protein G subunit alpha 11
HLA	human leukocyte antigen
H&E	haematoxylin and eosin
IHC	immunohistochemistry
LOH	loss of heterozygosity
MAPK	mitogen-activated kinase
mb	megabase
MDS	myelodysplastic syndrome
MEK	mitogen-activated protein kinase kinase
miRNA	micro ribonucleic acid
MLPA	multiplex ligation-dependent probe amplification
MFS	metastasis-free survival
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
n.a.	not available
NGS	next-generation sequencing
N/A	not available
OOG	Ocular Oncology group
PCR	polymerase chain reaction
PCLB4	phospholipase C beta 4
PUM	pediatric uveal melanoma
qPCR	quantitative polymerase chain reaction
RFS	recurrence-free survival

RNA	ribonucleic acid
ROMS	Rotterdam Ocular Melanoma Studygroup
SF3B1	splicing factor 3B subunit1
SNP	single nucleotide polymorphism
snRNP	small nuclear ribonucleoprotein
SNV	single nucleotide variations
SRSF2	splicing factor, arginine/serine-rich 2
TCGA	The Cancer Genome Atlas
TNM	Tumor Node Metastases
U2AF1	U2 small nuclear RNA auxiliary factor 1
UM	uveal melanoma
WGS	whole genome sequencing
wt	wildtype

Chapter 6.2

List of publications

van Poppelen NM*, van Ipenburg JA*, van den Bosch Q, Vaarwater J, Brands T, Eussen B, Magielsen F, Dubbink HJ, Paridaens D, Brosens E, Naus N, de Klein A, Kiliç E, Verdijk RM. Molecular Genetics of Conjunctival Melanoma and Prognostic Value of *TERT* Promoter Mutation Analysis. *Int J Mol Sci*. 2021 May 28;22(11):5784

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Chapter 6.3

About the author

Natasha Maria van Poppelen was born on October 4th 1988 in Woerden, The Netherlands. She graduated from secondary school in 2006 at Het Schoonhovens College in Schoonhoven. In 2006 she started medical school at the Erasmus Medical Center in Rotterdam. During medical school she went to Jakarta Indonesia under the supervision of Prof. Dr. P.M. van Hagen and Dr. R. La Distia Nora, and studied different types of uveitis and scleritis at the department of Ophthalmology at the Medical Faculty University of Indonesia. She spent 5 months in Tanzania during an internship in tropical medicine at St. Carolus Hospital. After obtaining her medical in degree in 2013 she worked almost two years at the department of Internal Medicine at the Vlietland Hospital in Schiedam. In September 2015, she was given the opportunity to start a PhD at the department of Clinical Genetics and Ophthalmology under the daily supervision of Dr. A. de Klein and Dr. E. Kiliç at the Erasmus Medical Center. During her PhD she focussed on the genetics of ocular melanoma, non-invasive testing and inheritance. This work is described in her thesis. In February 2019 she started as a resident in Ophthalmology at the department of Ophthalmology at Erasmus Medical Center headed by Prof. Dr. J.R. Vingerling.

Chapter 6.4

Phd Portfolio

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD candidate: N.M. van Poppelen
Erasmus MC Department: Ophthalmology & Clinical Genetics
Research School: Medical Genetics Centre South-West Netherlands (MGC)

PhD period: September 2015 – February 2019 (2022)
Promotor: Dr. J.E.M.M. de Klein
Supervisor: Dr. E. Kilic, Dr. N.C Naus

PhD training	**Year**	**Workload (ECTS)**
General courses		
- Modern Cancer Pathology Course, October 13 - 15, UMC Utrecht, Utrecht	2015	1.5
- SNPs and Human Diseases (MolMed), November 16 – 20, Erasmus MC, Rotterdam	2015	2.0
- Biomedical Research Techniques XIV (MolMed), November 2-6 November, Erasmus MC, Rotterdam	2015	1.5
- Research Integrity, February 25, Erasmus MC, Rotterdam	2016	0.3
- Basic and Translational Oncology (MolMed), October 10-14, Erasmus MC, Rotterdam	2016	1.8
- English for Academic purposes C1.1 Course, April 5 – June 6 2017, Erasmus University, Rotterdam	2017	2.5
- Biostatistical Methods I: Basic Principles (Nihes), September 18 – October 13, Erasmus MC, Rotterdam	2017	5.7
Specific courses (e.g. Research school, Medical Training)		
- Special topic course Chromatin (MGC), December 3, Erasmus MC, Rotterdam	2015	1.0
- Special topic course Signalling (MGC), March 3, Erasmus MC, Rotterdam	2016	1.0
- Genetics course (MGC), April 6 – May 31, Erasmus MC, Rotterdam	2016	3.0
- Safely working in the Laboratory, August 30, LUMC, Leiden	2017	0.3
- Special topic course Optogenetics (MGC), April 11 – May 23, Erasmus MC, Rotterdam	2018	2.0

Seminars and workshops

- CLC Workbench / Ingenuity Variant Analysis Workshop, 20 January, Erasmus MC, Rotterdam, the Netherlands	2016	0.5
- MGC PhD workshop, June 14 - 17, Dortmund, Germany	2016	1
- MGC PhD workshop, May 30 - June 2, Leuven, Belgium, "*Cell-free DNA as a non-invasive biomarker in uveal melanoma patients*" (poster presentation)	2017	1.5
- Microsoft Excel 2010: Basic workshop (MolMed), September 4, Erasmus MC, Rotterdam, the Netherlands	2017	0.3
- EndNote (Medische bibliotheek), November 10, Erasmus MC, Rotterdam	2017	0.15
- Photoshop and Illustrator CC 2018 workshop (MolMed), November 27, Erasmus MC, Rotterdam, the Netherlands	2018	0.3
- Photoshop and Illustrator CC 2018 follow-up workshop (MolMed), November 28, Erasmus MC, Rotterdam, the Netherlands	2018	0.3
- InDesign CC 2018 workshop (MolMed), December 21, Erasmus MC	2018	0.15

Presentations

- Bayer Ophthalmology Research Awards 2017, March 28, Maastricht, the Netherlands, "*Classifying UM patients bases on exosomal tumor DNA*", First prize winner €25,000.-	2017	0.3
- Clinical Genetics Research Meeting, May 9, Erasmus MC, Rotterdam, the Netherlands, "*Non-invasive techniques to classify uveal melanoma patients*"	2017	0.3
- Lunch meeting department of Ophthalmology, May 26, Erasmus MC, Rotterdam, the Netherlands, "*Classification of uveal melanoma patients using non-invasive techniques*"	2017	0.3
- Meeting department of Clinical Genetics & Ophthalmology, June 16, Erasmus MC, Rotterdam, Rotterdam, the Netherlands, "*Classification of uveal melanoma patients using non-invasive techniques*"	2018	0.3
- Melanoom Infodag, Stichting Melanoom, April 7, Amsterdam, the Netherlands, "*Prognostication of uveal melanoma patients*"	2018	0.3
- Clinical Genetics Research Meeting, May 22, Erasmus MC, Rotterdam, the Netherlands, *"Chromothripsis in uveal melanoma"*	2018	0.3

(Inter)national conferences

- 49th Ophthalmic Oncology group (OOG) Meeting, April 7-9, Athens, Greece	2016	1
- 26th Genetics Retreat – Nederlandse Vereniging voor Humane Genetica (NVHG) graduate meeting, April 21-22, Kerkrade, the Netherlands	2016	0.5
- Genome Informatics conference, September 19-22, Hinxton, United Kingdom, "*Chromosomal aberrations predict uveal melanoma mutations*" (poster presentation)	2016	1.5
- Cancer GenomiCs.nl (CGC.nl) annual meeting, Genomic Instability in Cancer, November 17-18, Amsterdam, the Netherlands	2016	0.5
- 51st Ophthalmic Oncology Group (OOG) Meeting, February 16-18, Rotterdam, the Netherlands, "*SRSF2 mutations in uveal melanoma*" (oral presentation)	2017	1.5
- International Society of Ocular Oncology (ISOO) Biennial Conference, March 24-28, Sydney, Australia, "*The genetic background of irismelanoma*" (oral presentation)	2017	1.5
- 211e Jaarvergadering Nederlands Oogheelkundig Gezelschap (NOG), March 29-31, Maastricht, the Netherlands, "*PRAME expression in uveal melanoma*" (oral presentation)	2017	1.5
- 27th MGC-Symposium, September 14, Rotterdam, the Netherlands	2017	0.3
- Cancer GenomiCs.nl (CGC.nl) annual meeting, New Horizons in Cancer Research, November 16-17, Amsterdam, the Netherlands	2017	0.5
- 212e Jaarvergadering Nederlands Oogheelkundig Gezelschap (NOG), March 21-23, Groningen, the Netherlands, "*The occurrence of chromothripsis in uveal melanoma*" (oral presentation)	2018	1.5
- 28th Genetics Retreat – Nederlandse Vereniging voor Humane Genetica (NVHG) graduate meeting, May 17-18, Kerkrade, the Netherlands, "*Are iris melanomas a different subgroup in uveal melanomas based on their genetic makeup?*" (oral presentation)	2018	1
- Keystone Symposium, Exosomes/Microvesicles: Heterogeneity, Biogenesis, Function and Therapeutic Developments, June 4-8, Breckenridge, United States, "*Is exosomal DNA useful for prognostication in uveal melanoma patients?*" (poster presentation)	2018	1.5

- European Human Genetics Conference (European Society of Human Genetics (ESHG) 52nd Meeting), June 16-19, Milan, Italy	2018	1
- 15th International Congress of the Society for Melanoma Research (SMR), October 24-27, Manchester, United Kingdom	2018	1
- NLSEV-2018 (Netherlands Society for Extracellular Vesicles), November 9, Amsterdam, the Netherlands	2018	0.3
- 7th Ocular Oncology Day – Basic Course of Ocular Oncology, December 6-7, Siena, Italy, "*Epigenetics & Non-invasive testing in uveal melanoma*" (oral presentation)	2018	0.5
- 213e Jaarvergadering Nederlands Oogheelkundig Gezelschap (NOG), March 27-29, Maastricht, The Netherlands, "*SRSF2 mutations in uveal melanoma*" (oral presentation)	2019	1.5
- 55th Ophthalmic Oncology Group (OOG) Meeting, April 11-13, London, United Kingdom, "*Pediatric Uveal Melanoma Study*" (oral presentation)	2019	1.5
- 215e Jaarvergadering Nederlands Oogheelkundig Gezelschap (NOG), March 24-26, virtual conference, The Netherlands, "*Germline BAP1 mutations in uveal melanoma*" (oral presentation)	2021	1.5
- 20th congress of the International Society of Ocular Oncology (ISOO), June 17-21, Leiden, The Netherlands, "*Genetics and prognostic value of TERT promoter mutations in conjunctival melanoma*" (oral presentation)	2022	1.5
- 216e Jaarvergadering Nederlands Oogheelkundig Gezelschap (NOG), June/July 29-1, Groningen, the Netherlands, "*Proton therapy in uveal melanoma patients from the Rotterdam Ocular Melanoma Studygroup*" (oral presentation)	2022	1.5
- 56th Ophthalmic Oncology Group (OOG) Meeting, October 19-22, Tel Aviv, Israel, "*Non-invasive testing in UM patients using circulating tumor DNA and shallow whole genome sequencing*" (oral presentation)	2022	1.5
- 10th Ocular Oncology Day, December 1-3, Siena, Italy, "*Family with mesothelioma and variant of unknown significance in the BAP1 gene*" (oral presentation)	2022	1.0

Other

- Clinical Genetics Research Meeting, weekly, Erasmus MC, Rotterdam, the Netherlands	2017-2019	1
- Lunch meeting department of Ophthalmology, weekly, Erasmus MC, Rotterdam, the Netherlands	2018-2019	1
- Clinical Genetics Journal Club, monthly, Erasmus MC, Rotterdam, the Netherlands	2018-2019	1

1. Teaching

- Supervising student Merel van Dijk, Hogeschool Rotterdam, Biologie en Medisch Laboratoriumonderzoek, August 29 – January 20	2016-2017	1
- Supervising student Shirin Mostert, Avans Hogeschool, Biologie en Medisch Laboratoriumonderzoek, February 6 – July 6	2017	1
- Supervising student Shirin Mostert, Avans Hogeschool, Biologie en Medisch Laboratoriumonderzoek, July 7 – December 28, afstudeerstage	2017	1
- Supervising student Luis Sanchez, Hogeschool Rotterdam February 5 – August 31	2018	1

2. Other activities

- Local organizing committee 51st Ocular Oncology Group (OOG) Meeting, Rotterdam, the Netherlands	2017	0.5
- Committee MGC PhD Workshop 2018, secretary, Texel, the Netherlands	2017-2018	1.5
- Committee Young Investigators Network Meeting (YIN) 55th Ocular Oncology Group (OOG) Meeting, London, United Kingdom	2019	0.5
- Committee Young Investigators Network Meeting (YIN) 56th Ocular Oncology Group (OOG) Meeting, Tel Aviv, Israel	2022	0.5

3. Awards

- Bayer Ophthalmology Research Awards 2017, First prize winner €25,000.-	2017	

Total ECTS **64.2**