Genetic prognostication in uveal melanoma

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Chapter 3

Gender and chromosome 8q status influence survival in Uveal Melanoma patients surviving more than five years following enucleation

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ABSTRACT

Importance: A subgroup of uveal melanoma (UM) runs an indolent course and gives rise to clinical metastases at a late stage. Identifying patient features and tumor characteristics associated with late metastasis is important for patient counselling and planning of adjuvant therapies.

Objective: To identify patient and tumor characteristics that influence UM-related death more than five years following enucleation.

Design: A retrospective cohort study of 586 primary UM, enucleated between March 1983 and December 2013 at the Leiden University Medical Center, The Netherlands.

Setting: A single-center study at a national referral center for UM.

Participants: Patients with UM who have undergone a primary enucleation.

Main Outcomes and Measures: Distribution of patient and tumor characteristics in three groups of patients who died due to UM metastases in different periods: 1) in the first three years; 2) between three and five years; 3) more than five years after enucleation. Regression analyses with UM-related death as endpoint in the total cohort and in those surviving longer than five years following enucleation.

Results: Patients dying of UM more than five years after enucleation were often female (p=0.01), of a younger age (p=0.001), and had tumors with a spindle cell type (p<0.001), stage I AJCC classification (p=0.02), absence of monosomy 3 (p=0.003), a smaller diameter (p=0.03), and lower mitotic count (p=0.01) than those dying earlier.

Increasing tumor diameter (HR: 1.11, 95% CI: 1.02 – 1.21, p=0.02) and mitotic count (HR: 1.08, 95% CI: 1.04 – 1.12, p<0.001), presence of extravascular matrix loops (HR: 2.13, 95% CI: 1.13 – 4.73, p=0.02), and gain of 8q (HR: 3.97, 95% CI: 1.96 – 8.04, p<0.001) were independently associated with UM-related death throughout the whole follow-up period. After five years of follow-up, only female gender (HR: 0.23, 95% CI: 0.06 – 0.83, p=0.02) and gain of 8q (HR: 21.37, 95% CI: 2.56 – 178.52, p=0.005) were independently related to UM-related death.

Conclusions and Relevance: Patients who die due to UM metastases at a late stage are more often women and younger patients and less often exhibit tumor features characteristic of a high malignancy grade. Gender and chromosome 8q status are the only factors independently influencing survival in patients surviving more than five years after enucleation.

INTRODUCTION

Uveal melanoma (UM) is the most common type of ocular melanoma and originates from melanocytes residing in the uveal tract.[1] It is the most frequently-occurring primary intraocular malignancy in adults and is predominantly found in Caucasians, especially in those with fair skin and light iris color. The mean annual age-adjusted incidence is 5.1 per million in the United States.[2] It is estimated that 6,700 to 7,100 new patients are diagnosed worldwide annually and that there are 87,000 to 106,000 survivors, many of whom are under surveillance for the development of overt metastases.[3] UM metastasizes hematogenously, with a predilection for the liver. Up to 50% of patients die due to metastases within 10 years after diagnosis.[4] Once metastases have developed, the median survival time ranges from 4 to 15 months.[5,6]

Clinical and histopathologic tumor characteristics such as largest basal diameter (LBD), thickness, ciliary body (CB) involvement, extraocular growth (which together determine the American Joint Committee on Cancer (AJCC) Tumor, Node, Metastasis (TNM) stage), mitotic count, cell type, and extravascular matrix loops have been related to the development of metastases.[7-10] Additionally, genomic non-random chromosome alterations resulting in monosomy 3 and gain of 8q, and a specific class 2 gene-expression profile are associated with the occurrence of metastatic disease.[11-13] Recently, mutations in specific genes such as *BAP1*, *EIF1AX*, and *SF3B1* have been shown to have prognostic value in UM.[14-17] Besides tumor characteristics, patient parameters like gender and age may play a role in survival in UM; males have been shown to have earlier and more frequent metastases in the first decade after diagnosis, while a lower rate of metastasis has been reported in younger patients.[18-20]

The moment disseminated disease is detected and leads to death differs considerably between patients.[21] Some UM patients (about 1.5%) have detectable metastases at the time of diagnosis of the primary tumor, whereas others develop overt metastases more than 10 years after diagnosis.[22,23]

The variability in survival of patients with metastasizing UM may be explained by diversity in aggressiveness of metastasized tumor cells or robustness of host immune responses. Metastases of UM vary in aggressiveness, as evidenced by analysis of their doubling times, which have been estimated to range from 34 to 220 days.[24] An alternative explanation is lead-time bias: some patients with larger primary tumors seem to have a shorter survival because their tumors are detected late and have been growing and metastasizing for a longer period of

time.[25] Considering survival after detection of metastases, patients undergoing surveillance for metastases may seem to live longer after detection of metastases because their metastases have been detected earlier.[26]

We hypothesize that not only the size of the primary tumor, which reflects a combination of the tumor's growth rate and lead-time bias, but also other histopathologic and genetic characteristics of the primary tumor and patient parameters are associated with the rate of growth of micrometastases to clinically detectable ones and thus the time-to-death due to metastases.

We assume that patients who have died due to UM metastases after a considerably long period differ in gender, age, and tumor characteristics from patients dying early after treatment of the primary tumor. To study our hypothesis, we analyzed the association of patient characteristics and primary tumor features with survival in a cohort of 586 patients who had undergone primary enucleation for UM in our center.

METHODS

Patients

Between March 1983 and December 2013, a total of 586 patients with UM underwent a primary enucleation at the Leiden Universiy Medical Center, the Netherlands. Baseline characteristics are shown in Table 1. Although we have kept a registry of enucleated UM patients since 1972, patients who underwent an enucleation before 1983 (the era preceding brachytherapy) were not included in the current study, since they may bias the results due to smaller tumor sizes. For this retrospective cohort analysis, we did not include patients who had undergone brachytherapy prior to enucleation, since this may influence UM histopathology and genetics.[27-29]

The cause and date of death were obtained from the Integral Cancer Center West (a regional office of the Netherlands Comprehensive Cancer Organisation, https://iknl.nl/over-iknl/about-iknl). Every Dutch cancer patient is reported to the Netherlands Comprehensive Cancer Organisation by their general physician. Physicians employed by this institution are responsible for collecting information on survival status of cancer patients by contacting the general physician, who registers the date and cause of death of his/her patients.

We do not have information on the date of detection of overt metastases, as this was often not registered. Follow-up time, defined as the time period between primary enucleation and death or last date of follow-up, was updated in March

2017. The mean follow-up time was 5.1 years, at which time 213 patients had died due to UM metastases and 140 due to other causes, whereas 233 were alive (Table 1). Seven patients were lost to follow-up because of emigration.
The Ethics Committee of the LUMC waived the need for approval of this retrospective study, which followed the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964, ethical principles for medical research involving human subject).

Histopathologic examination

After enucleation, a portion of the tumor was snap frozen and stored at -80 °C. The remaining tumor was fixed in 4% neutral-buffered formalin for 48 hours and embedded in paraffin. Haematoxylin and eosin-stained 4-µm thick sections were analyzed by an ocular pathologist using a standard protocol for determination of histopathologic characteristics: LBD (in millimeters), thickness (apical height, in millimeters), mitotic count (per 2 mm2 at 40x magnification), tumor location, cell type (assessed as described in the Armed Forces Institute of Pathology atlas[30]; 'mixed' when at least 5% of both spindle and epithelioid cells are present, otherwise 'spindle' or 'epithelioid'), and (since January 1991) the presence of extravascular matrix loops (which by definition includes networks) determined on periodic acid-Schiff (PAS)-stained slides without a counterstain and without a filter.[31] Tumors were staged in accord with the 7th edition of the AJCC Cancer Staging Manual.[7,32]

Cytogenetic analysis

Between 1999 and 2013, 291 UM samples were sent for cytogenetic analysis by karyotyping with or without fluorescence *in situ* hybridization (FISH). In 62 of these and 4 other tumors, single nucleotide polymorphism (SNP) analysis has been performed.[33]
GTG-banded (G-banding with Giemsa and trypsin) metaphases were used for karyotyping. Karyograms were analyzed using the automatic karyotyping software Cytovision (Leica Biosystems Inc., Buffalo Grove, IL, USA) and described using the regulations of the International System for Human Cytogenetic Nomenclature (1995). Loss of a copy of chromosome 3 or gain of chromosome 8q in at least two cells was sufficient to classify the tumor as having the abnormality. If monosomy 3 was present in only one cell, the tumor was designated as a monosomy 3 tumor provided that other chromosome abnormalities common in UM were present. In

case of 8q gain, the presence of an isochromosome 8q in only one cell was sufficient.

FISH was performed using DNA-probes specific for the centromere of chromosome 3 (probe: α-sat3, Cytocell, Cambrigde, UK) and region 3p24.3-p25 (probe: RP11-322M13, Cytocell). Monosomy 3 or chromosome 8q status was assigned if this aberration was present in at least 10% of the analyzed cells.

The Affymetrix 250K_NSP chip and the Affymetrix Cytoscan HD chip were used for SNP analyses. Copy numbers were determined using the "Genotyping Console (GTC)" and the "GTC Browser" to visualize the data in the analysis of the Affymetrix 250K_NSP chips (both from Affymetrix, Santa Clara, CA, USA). Affymetrix Cytoscan HD chips were analyzed using "Chromosome Analysis Suite (ChAS)". Different loci per chromosome were analyzed to adjust for partial gains or deletions. Approximately 200 probes per gene locus were averaged to determine copy numbers.

In cases of discrepancy between the tests, the tumor was classified as having monosomy 3 or chromosome 8q gain when either of the tests showed the abnormality.

Statistical analysis

Data from clinical charts, pathology reports, genetic tests and information on follow-up status were transferred to a database for evaluation with the statistical software package SPSS (IBM SPSS Statistics for Windows, version 20.0.0, Armonk, NY: IBM Corp.).

To evaluate whether patients who die early or late due to UM metastases differ in patient and tumor characteristics, we stratified patients who died due to UM metastases in three groups based on follow-up time (three years, between three and five years, more than five years) and analyzed differences in patient and tumor characteristics. Three and five years were chosen as cut-off values since these follow-up periods are commonly reported in epidemiologic studies in oncology. Characteristics were compared between the three groups using the Pearsons's chi-square test for nominal categorical variables, the Linear-by-linear Association test for an ordinal categorical variable, and the Kruskal-Wallis test for numerical parameters.

Cox regression analyses were conducted in order to identify factors influencing survival. Effect estimates are reported as hazard ratios (HRs) with 95% confidence intervals (CI) of death due to UM metastases by censoring for end of follow-up or death due to other causes. Besides unadjusted univariate analyses, multivariable

regression analyses were performed to evaluate independent effects. To evaluate possible differences in results due to the effect of deaths due to other reasons than UM metastases, competing risks regression analyses based on the Fine-Gray model were conducted and cumulative incidence curves (package *cmprsk*) were generated and compared with the Gray's K-sample test using the statistical software package *R* (version 3.4.0).[34-36] These analyses take other reasons of death into account as competing risks, which are treated as censored observations in conventional statistics such as Cox regression and Kaplan-Meier analyses. Censoring other causes of death possibly exaggerates the apparent metastatic death rate.[21] Regression analyses were performed in the total cohort of 586 patients as well as in those who survived, with or without metastases, more than five years after enucleation (n=299). The competing risks regression model is the main regression analysis, while the Cox regression model is included for ease of interpretation. All statistical tests were two-sided and a p-value < 0.05 was considered to be statistically significant.

RESULTS

Population characteristics

In our cohort of 586 UM patients, 53% were male. Median age at time of enucleation was 62.5 years (Table 1). The median tumor diameter was 12 mm and median tumor thickness was 6 mm. The median mitotic count was 4 per 2 mm^2. Almost one-third of cases showed ciliary body involvement. A mixed/epithelioid cell type was recorded in 70% of cases, while 65% of tumors had extravascular matrix loops. Most tumors (60%) were classified as AJCC stage II. Monosomy 3 was detected in 53% of the cases, while 47% harbored gain of chromosome 8q. At last follow-up (median: 5.1 years), 236 (40%) of patients were diagnosed with clinical UM metastases and 213 (36%) had died due to UM metastases.

Characteristics of patients who died due to UM metastases in relation to survival time

In order to determine whether different patient and tumor characteristics are associated with early or late death from UM, we performed a comparative analysis of three different follow-up periods in a subgroup of 225 patients: three years ('early-death group'), three to five years ('intermediate-death group'), and more than five years ('late-death group') follow-up after enucleation (Table 2). This cohort consisted of 213 patients who died due to UM and 12 patients with metastasized UM who were alive or died due to other causes after more than five

years of follow-up. The latter group was included since these patients have UM metastases and have 'proven' to survive at least five years. Patients with UM

Table 1. Characteristics of the total cohort of UM patients primarily enucleated in the LUMC between 1983 and 2013 (n=586). Percentages, which are reported after exclusion of missing data, have been rounded and may not total 100.

Characteristic	*n*	%/range
Gender		
Female	273	(47%)
Male	313	(53%)
Age at enucleation (years)		
Median	62.5	(7.0 – 91.3)
Largest basal diameter (mm)		
Median	12.0	(0.4 – 30)
Thickness (mm)		
Median	6.0	(0.5 – 17)
Mitotic count		
Median	4.0	(0 – 35)
Ciliary body involvement	177	(30%)
Cell type		
Spindle	175	(30%)
Mixed/Epithelioid	409	(70%)
Extravascular matrix loops	247	(65%)
AJCC Stage		
I	113	(20%)
II	342	(60%)
III	119	(21%)
Monosomy 3 *(determined in 275 cases)*	146	(53%)
Gain of 8q *(determined in 245 cases)*	115	(47%)
Follow-up time (years)		
Median	5.1	(0.08 – 33.3)
Metastases	236	(40%)
Vital status		
Death due to UM metastases	213	(36%)
Death due to other causes	140	(24%)
Alive at last follow-up date	233	(40%)

metastases and shorter than five year follow-up who were alive or dead due to other causes were excluded since it is unknown when these patients (eventually) would have died due to UM metastases.

Patients of the early-death group were significantly more often males than those of the intermediate-death group (56% vs 32%, p=0.01), while the late-death

Table 2. Clinicopathologic and genetic tumor characteristics of patients who died due to UM metastases, stratified into three groups according to the time period between enucleation and death: 1) three years 2) between three and five years 3) more than five years. The group of patients who died after five years also comprises 12 patients with metastasized UM who were alive or died due to other causes after five years of follow-up. Percentages are rounded and may not total 100. Significant p-values are in bold.

Characteristic	Time period						
	< 3 years (*n*=102)		3 - 5 years (*n*=59)		> 5 years (*n*=64)		*P* value
Gender							
Female	45	(44%)	40	(68%)	30	(47%)	
Male	57	(56%)	19	(32%)	34	(53%)	**0.01***
Age at enucleation (years)							
Median (range)	66.4	(27.4 – 89.6)	63.6	(25.6 – 82.8)	60.1	(28.7 – 87.7)	**0.001**†
Largest basal diameter (mm)							
Median (range)	14.0	(0.4 – 30.0)	13.0	(5.0 – 22.0)	12.0	(3.0 – 24.0)	**0.03**†
Thickness (mm)							
Median (range)	7.0	(0.5 – 15.0)	7.0	(1.0 – 12.0)	7.0	(0.8 – 15.0)	0.68†
Mitotic count							
Median (range)	6	(1– 33)	6	(1 – 35)	5	(0 – 30)	**0.01**†
Ciliary body involvement	51	(50%)	22	(37%)	21	(33%)	0.07*
Cell type							
Spindle	9	(9%)	10	(17%)	26	(41%)	
Mixed/Epithelioid	92	(91%)	48	(83%)	38	(59%)	**<0.001***
Extravascular matrix loops *(known in 136 cases)*	56	(86%)	31	(82%)	24	(73%)	0.26*
AJCC Stage							
I	8	(8%)	6	(10%)	10	(16%)	
II	51	(51%)	35	(60%)	36	(57%)	
III	42	(42%)	17	(29%)	17	(27%)	**0.02**‡
Monosomy 3 *(known in 101 cases)*	52	(90%)	15	(68%)	12	(57%)	**0.003**†
Gain of 8q *(known in 88 cases)*	39	(80%)	15	(71%)	12	(67%)	0.51†

*Symbols: * Pearson's chi-square test, † Kruskal-Wallis test, ‡ Linear-by-Linear Association test*

group was significantly younger at enucleation than the others (60 versus 64 in the intermediate-death group and 66 in the early-death group) (p=0.001). The tumors of patients in the late-death group had a smaller diameter than tumors in the intermediate-death and early-death group (12 versus 13 and 14 mm, respectively, p=0.03) and lower mitotic count (5 versus 6 in the other groups,

p=0.01), while no difference in thickness was noted. Furthermore, the late-death group tended to have less frequent ciliary body involvement (p=0.07), a less frequent mixed/epithelioid cell type (p<0.001), more AJCC Stage I tumors (p=0.02), and a much lower occurrence of monosomy 3 (p=0.003). Although gain of 8q was also least common in the late-death group, the difference was not statistically significant (p=0.51). The chromosome 8q status was, however, known in only 88 of the 225 cases in this analysis, while the chromosome 3 status was known in 101 tumors.

Regression analyses

Cox regression and competing risks regression analyses were conducted in the total cohort as well as in patients with more than five years follow-up (Table 3). In the total cohort, the competing risks regression model showed increasing tumor diameter (HR: 1.11, 95% CI: 1.02 – 1.21, p=0.02) and mitotic count (HR: 1.08, 95% CI: 1.04 – 1.12, p<0.001), the presence of extravascular matrix loops (HR: 2.31, 95% CI: 1.13 – 4.73, p=0.02), and gain of 8q (HR: 3.97, 95% CI: 1.96 – 8.04, p<0.001) to be significantly associated with UM-related death (Table 3A). Except for age at enucleation which was associated with the risk of death due to UM metastases in the Cox regression model, the results of both models were in concordance.

When analyzing patients who survived more than five years after enucleation, only female gender (HR: 0.23, 95% CI: 0.06 – 0.83, p=0.02) and gain of 8q (HR: 21.37, 95% CI: 2.56 – 178.52, p=0.005) were independently associated with UM-related death in the competing risks regression model (Table 3B). The multivariable Cox regression model identified gain of8q as the only parameter significantly associated with death due to UM metastases.

To visualize the effect of gender and chromosome 8q status on patient survival after five years following enucleation, we generated cumulative incidence curves which take competing risks into account (Figure 1). Cumulative incidence curves did not show a significant difference in incidence of UM-related death between men and women (p=0.78) (Figure 1A), while gain of 8q is associated with poor survival throughout the whole follow-up period (p<0.001) (Figure 1B).

Although cumulative incidence curves showed monosomy 3 to be significantly associated with a higher incidence of UM-related death after five years (p=0.03) (Figure 1C), the regression analyses did not identify it as a factor independently

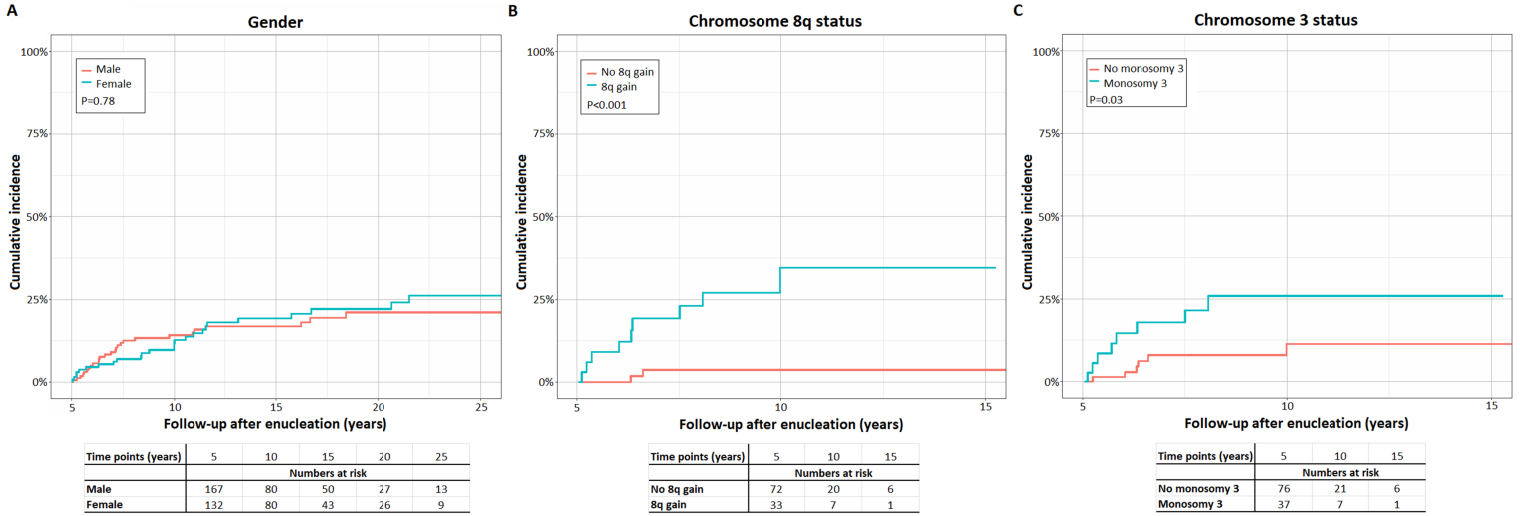
influencing survival in cohort of patients surviving longer than five years after enucleation (Table 3B).

Table 3. Cox regression and competing risks regression analyses with death due to UM metastases as the endpoint of interest, evaluating the effect of patient and tumor characteristics on survival in the total cohort and in those who were still alive after 5 years. A: in the total cohort (n=586), B: after five years follow-up (n=299). Significant p-values are in bold.
A:

Characteristic	**Univariate Cox regression**			**Multivariable Cox regression**			**Multivariable Competing risks regression**		
	HR	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Female gender	1.18	0.9 – 1.54	0.23	0.59	0.35 – 1.0	0.05	0.67	0.4 – 1.13	0.13
Age at enucleation	1.03	1.02 – 1.04	**<0.001**	1.03	1.0 – 1.05	**0.02**	1.02	0.99 – 1.04	0.15
Largest basal diameter	1.16	1.12 – 1.2	**<0.001**	1.11	1.02 – 1.2	**0.02**	1.11	1.02 – 1.21	**0.02**
Thickness	1.08	1.03 – 1.12	**0.001**	0.91	0.82 – 1.0	0.05	0.91	0.81 – 1.01	0.08
Mitotic count	1.06	1.04 – 1.08	**<0.001**	1.07	1.03 – 1.12	**0.001**	1.08	1.04 – 1.12	**<0.001**
Ciliary body involvement	2.13	1.61 – 2.81	**<0.001**	1.15	0.63 – 2.12	0.65	1.03	0.59 – 1.81	0.91
Mixed/Epithelioid cell type	2.51	1.78 – 3.54	**<0.001**	1.11	0.53 – 2.34	0.78	1.12	0.61 – 2.06	0.71
Extravascular matrix loops *(known in 379 cases)*	2.98	1.9 – 4.69	**<0.001**	2.41	1.11 – 5.26	**0.03**	2.31	1.13 – 4.73	**0.02**
AJCC Stage I *(reference category)*	-	-	-	-	-	-	-	-	-
AJCC Stage II	1.94	1.25 – 3.0	**0.003**	1.58	0.33 – 7.62	0.57	1.42	0.41 – 4.93	0.58
AJCC Stage III	4.78	3.0 – 7.6	**<0.001**	2.57	0.44 – 14.88	0.29	2.52	0.59 – 10.85	0.21
Monosomy 3 *(known in 275 cases)*	5.42	3.26 – 9.01	**<0.001**	1.65	0.76 – 3.55	0.21	1.49	0.73 – 3.02	0.27
Gain of 8q *(known in 245 cases)*	5.92	3.5 – 10.02	**<0.001**	4.82	2.32 – 9.99	**<0.001**	3.97	1.96 – 8.04	**<0.001**

B:

Characteristic	**Univariate Cox regression**			**Multivariable Cox regression**			**Multivariable Competing risks regression**		
	HR	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Female gender	1.03	0.6 – 1.77	0.93	0.23	0.03 – 1.77	0.16	0.23	0.06 – 0.83	**0.02**
Age at enucleation	1.03	1.0 – 1.05	**0.03**	1.04	0.96 – 1.12	0.37	1.03	0.96 – 1.1	0.48
Largest basal diameter	1.13	1.07 – 1.22	**<0.001**	1.18	0.85 – 1.64	0.33	1.12	0.81 – 1.55	0.49
Thickness	1.09	1.0 – 1.18	0.05	0.81	0.57 – 1.16	0.25	0.84	0.47 – 1.51	0.57
Mitotic count	1.02	0.97 – 1.07	0.42	1.21	0.93 – 1.57	0.15	1.22	0.85 – 1.76	0.27
Ciliary body involvement	1.49	0.78 – 2.85	0.23	0.65	0.02 – 19.42	0.81	0.69	0.0 – 619.09	0.92
Mixed/Epithelioid cell type	1.13	0.65 – 1.96	0.66	0.33	0.04 – 2.94	0.32	0.36	0.04 – 3.44	0.38
Extravascular matrix loops *(known in 177 cases)*	2.1	0.88 – 5.04	0.1	7.76	0.55 – 108.67	0.13	9.62	0.88 – 104.64	0.06
AJCC Stage I *(reference category)*	-	-	-	-	-	-	-	-	-
AJCC Stage II	1.56	0.76 – 3.2	0.23	1.29	0.04 – 41.42	0.89	1.45	0.04 – 46.67	0.84
AJCC Stage III	3.32	1.45 – 7.58	**0.004**	6.81	0.05 – 859.41	0.44	7.47	0.01 – 7818.89	0.57
Monosomy 3 *(known in 113 cases)*	3.12	1.08 – 9.02	**0.04**	1.27	0.19 – 8.51	0.81	1.24	0.21 – 7.44	0.82
Gain of 8q *(known in 105 cases)*	10.31	2.23 – 47.74	**0.003**	21.25	2.41 – 187.49	**0.006**	21.37	2.56 – 178.52	**0.005**



Time points (years)	5	10	15	20	25
	Numbers at risk				
Male	167	80	50	27	13
Female	132	80	43	26	9

Time points (years)	5	10	15
	Numbers at risk		
No 8q gain	72	20	6
8q gain	33	7	1

Time points (years)	5	10	15
	Numbers at risk		
No monosomy 3	76	21	6
Monosomy 3	37	7	1

Figure 1. Cumulative incidence curves showing the effect of gender (A), chromosome 8q status (B), and chromosome 3 status (C) on incidence of UM-related death.

DISCUSSION

This retrospective cohort study in 586 patients demonstrates that tumors of patients who die due to UM metastases more than five years after enucleation less often exhibit characteristics of high malignancy than those who die earlier, and shows that gender and chromosome 8q status are the only factors that continue to be associated with survival, as shown after five years following enucleation. The study confirms known associations between several features of primary UM and survival.[37]

Various factors which have previously been identified as prognostic indicators in UM show significant differences in distribution between patients who died early and those who died late due to UM-related disease. Patients who died relatively late due to UM metastases were significantly more often women and were younger at the time of enucleation; their tumors had smaller diameters and a lower mitotic count, were more often AJCC stage I tumors and had a spindle cell type. They were also more often posteriorly located, although the difference was only borderline significant. Regarding genetic features, monosomy 3 was found in 57% and gain of 8q in 67% of patients who died after 5 years of follow-up versus 90% and 80%, respectively, in patients who died during the first three years after enucleation.

Earlier studies have reported on patient and tumor characteristics of long-term metastatic UM survivors and factors influencing long-term prognosis of UM patients.[21-23] Rietschel et al. found that female gender and younger age at diagnosis of metastatic melanoma were predictive of a prolonged survival in patients with metastatic uveal melanoma and a later study by Buzacco et al. corroborated the association of young age with prolonged survival in metastatic uveal melanoma.[22,23] Comparable results were reported in a large study by Shields et al., in which older patients were found to die of metastases significantly more often than their younger counterparts, and by Damato et al. who reported larger tumors with a higher malignancy degree in older patients .[19,20] However, Kujala et al., who performed multivariate competing risks regression analyses (which take competing risks of death into account), found no influence of age and a borderline effect of gender on survival in a cohort of 289 primary UM with long-term follow-up.[21] In accordance with the study by Kujala et al., our competing risks regression analysis showed no effect of age at enucleation on survival and demonstrated that gender influences survival in those that survive the first five years after enucleation.

Male gender has been associated with increased risk of death from all causes and with worse survival after metastases have developed.[22,37] A study by Zloto et al. showed that male gender predicted earlier and more frequent metastases in the first decade after the diagnosis and also found gender to be important for the rate at which metastases appear and their apparent aggressiveness.[18] A recent study in 344 South Korean UM patients found higher survival probabilities for females with UM.[38]

Several explanations can be proposed for the observed association of male gender with poor survival in our study, which was evident after five years of follow-up in our competing risks regression analysis. One possibility is that males might have larger and more advanced primary tumors than females, as described in some but not all reports.[18,39] The larger tumor size in men could be due to tumors of a higher grade of malignancy or a delay in diagnosis and treatment (lead-time bias) in males.[18,40] However, male gender was independently associated with poor survival by multivariate competing risks regression analysis after five years of follow-up. A more likely explanation for worse survival of males could be their potentially poorer general health, making them more vulnerable than women to metastases of comparable malignancy grades. A third explanation could be a contributing effect of testosterone on growth of micrometastases or a less efficient immune surveillance on the outgrowth of metastases in men.[18] This might be related to the differential way sex hormones interact with the immune system. Likewise, males and females are known to differ for instance in their risk of autoimmune diseases.[41] However, the exact role of gonadal hormones in UM survival remains unclear. Studies that have explored the potential role of female hormones in the etiology of UM and the risk of metastases have found no evidence of such a relationship.[42-44]

It is remarkable that gender only influenced survival after five years of follow-up. We propose that these tumors represent a fairly homogenous group of cases with a low malignancy grade in which the effect of gender on survival is probably enlarged.

Older age at enucleation was associated with early UM-related death and influenced survival in the total cohort according to the Cox regression analysis. However, the competing risks regression analysis did not identify age as an inpendent factor influencing survival, suggesting that the independent association of older age and worse survival in our Cox regression analysis and in earlier

studies is based on biases induced by ignoring competing risks as proposed previously by Kujala et al.[21]

Anatomic and histologic tumor features characteristic of higher aggressiveness (large tumor size, high mitotic count, ciliary body involvement, mixed/epithelioid cell type, higher AJCC stage) were less frequent in patients dying of UM metastases late compared to early. However, only large tumor diameter, high mitotic count and the presence of extravascular matrix loops were independently associated with poor survival in the total cohort. Remarkably, none of the anatomic and histologic tumor parameters influenced survival after five years of follow-up. As suggested above, primary UMs possessing these characteristics may lead to more aggressive metastases that leads to death early on, after which a more homogenous group of patients remains in which these factors have a diminishing effect and survival probability is more strongly determined by gender and chromosome status. However, it is noteworthy to mention that the presence of extravascular matrix loops showed borderline significant (p=0.06) association with survival after five years following enucleation in the multivariable competing risks regression model.

Gain of 8q was the parameter with the highest HR independently associated with poor survival in the total cohort as well as specifically after five years following treatment of the primary tumor. It is appealing to assume that tumors with more than five years of follow-up may more often be disomy 3 tumors, in which gain of 8q is a strong predictor of poor outcome. The late-death cases may show disomy 3/class 1 with overexpression of *PRAME* or *SF3B1* mutation.[15,45,46] Recently, *PRAME* overexpression has been shown to be associated with metastatic risk in class 1 tumors and the *SF3B1* mutation is similarly associated with the development of metastases in disomy 3 tumors.[45,46] As we do not know the mutation status of our tumors, we cannot compare survival with different types of mutations.[46]

Remarkably, chromosome 3 status was neither throughout the whole follow-up period nor after five years of follow-up independently associated with prognosis, although cumulative incidence curves show that monosomy 3 was associated with a significantly higher incidence of UM-related death after five years following enucleation (Figure 1C). We hypothesize that the effect of monosomy 3, which has been shown to be strongly correlated to histopathologic tumor features, on survival was decreased by the presence of anatomic and histologic tumor features that we have evaluated in our model. This may be especially true for extravascular

matrix loops, which have been demonstrated to correlate strongly with monosomy 3, and may even be used to predict monosomy 3 accurately in 70% of cases when considered together with cell type as shown in a study by Sandinha et al.[47,48] In accordance with this, we did find an independent effect of chromosome 3 status on survival in the total cohort as well as specifically after five years of follow-up, if we excluded extravascular matrix loops from our regression model (data not shown). Nevertheless, our data corroborates the strong correlation between extravascular matrix loops and monosomy 3 as previously shown, and demonstrates that gain of 8q is a stronger predictor of survival time than chromosome 3 status in our cohort, when these two genetic prognostic markers are evaluated in combination with patient features and anatomic and histologic tumor characteristics. Although monosomy 3 is a strong predictor of metastatic death, it may be a less strong predictor of time to metastatic death.[48] As suggested earlier, factors that regulate metastatic spread may not be strong determinants of survival time.[4,21]

The major strength of our study is that our cohort of patients with histopathological and genetic data has a long follow-up period. Although the correlation of predictive factors with time to metastatic death has been shown before,[20,37] we have performed competing risks regression analysis to take into account competing risks of death and specifically analyzed factors influencing survival in long-survivors. However, because morbidity and mortality from non-malignant diseases, which form the main competing risk of death of patients with posterior UM,[7] vary between countries, our results must be confirmed in other populations. Mortality from other causes influences which patients live long enough to develop evidence of metastatic UM. Gender and age are variables that can be prominently influenced by competing risks.[21]

A limitation of our study is that we have restricted our study to patients who underwent primary enucleation, which makes our results not applicable to patients undergoing eye-preserving treatment options. Another limitation is the fact that chromosome 3 and 8q status was not known in approximately half of the patients and was determined by karyotyping and FISH in most of the cases. We believe that the true percentage of tumors having monosomy 3 or gain of 8q may be higher when determined by newer and more sensitive techniques such as SNP-based copy number determination. We started performing SNP-based analyses in our clinic in September 2015.

In conclusion, our study corroborates that the subgroup of patients who die because of UM metastases at a relatively late stage following treatment of the primary tumor are more often females and younger at enucleation, and less often exhibit tumor features characteristic of high malignancy when compared to those dying early. Furthermore, we demonstrate that in long-survivors, only male gender and gain of 8q are independent predictors of poor outcome. The findings of our study have implications regarding counselling of patients who have survived more than five years following enucleation. The fact that an indolent subtype of UM, such as the class 1 tumors, that gives rise to clinical metastases at a later stage exists, should be kept in mind when counselling these long-survivors. Gender and chromosome 8q status seems to affect melanoma-specific survival in these patients, and should be taken into account, possibly together with extravascular matrix loops, for counselling of these patients. Furthermore, identification of characteristics of patients who die at a later stage may help identifying candidates who may benefit the most from adjuvant therapies since these patients have slow growing metastases, in contrast to patients who have aggressive metastases and die early on leaving no possibilities for starting adjuvant therapy. Future studies in larger cohorts that include information on chromosome status, gene-expression profiling and specific genes such as *SF3B1* and *PRAME* are necessary to accurately predict patients who may develop metastases at a later stage and to enhance prognostication in long-term survivors.

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Competing Interests Statement

All authors declare that no conflicts of interest exist.

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