Discovering biomarkers and druggable targets in uveal melanoma

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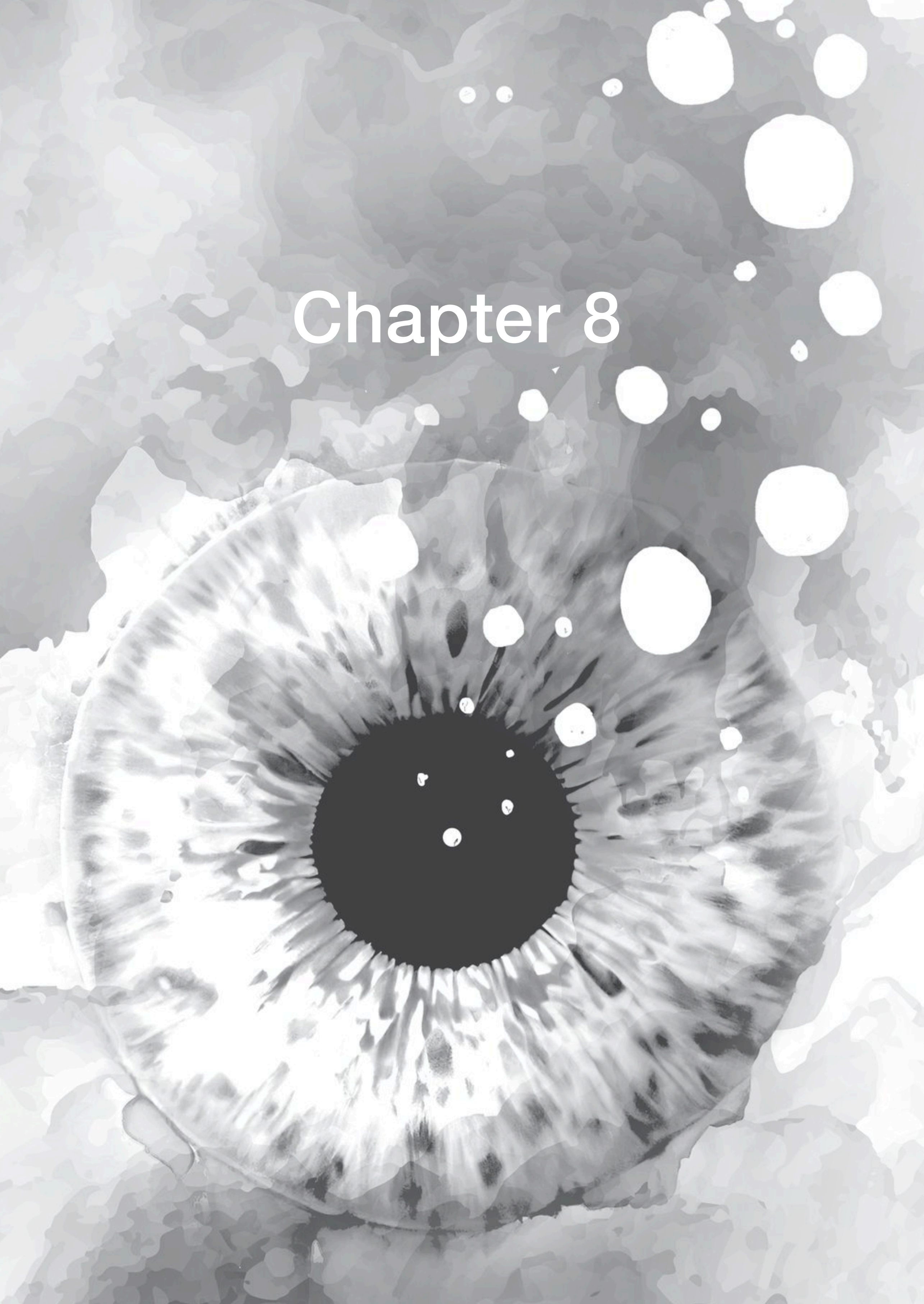
Chapter 8



Chromosome 3 and 8q aberrations in Uveal Melanoma show greater impact on survival in patients with light iris versus dark iris color.

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Abstract

Purpose: Individuals with a gray, blue, or green iris have a higher chance of developing uveal melanoma (UM) than those with brown eyes. We wondered whether iris pigmentation might not only be related to the predisposition to UM, but also to its behavior and therefore compared clinical, histopathologic, and genetic characteristics of UM between eyes with different iris colors.

Design: We determined iris color in a large cohort of patients who had undergone an enucleation for UM. Clinical and histopathological tumor characteristics, chromosome status, and survival were compared between three groups, based on iris color.

Participants: 412 patients with choroidal/ciliary body UM, who had undergone a primary enucleation at the Leiden University Medical Center (LUMC), Leiden, The Netherlands, between 1993 and 2019, divided into three groups, based on iris color (gray/blue, green/hazel, and brown). Validation cohort: 934 choroidal/ciliary body UM patients treated at Wills Eye Hospital (WEH), Philadelphia, United States.

Methods: A comparison of clinical, histopathologic, and genetic characteristics of UM in patients with different iris colors.

Main Outcome Measures: Melanoma-related survival in UM patients, divided over three iris color groups, in relation to the tumor's chromosome 3 and 8q status.

Results: Moderate and heavy tumor pigmentation was especially seen in eyes with a brown iris ($p < 0.001$). Survival did not differ between patients with different iris colors ($p = 0.28$). However, in the patients with a light iris, copy number changes in chromosome 3 and 8q had a greater influence on survival than in patients with a dark iris. Similarly, chromosome 3 and chromosome 8q status affected survival more among patients with lightly-pigmented tumors than in patients with heavily-pigmented tumors. The WEH cohort similarly showed a greater influence of chromosome aberrations in light-eyed individuals.

Conclusions: While iris color by itself did not relate to survival of UM patients, chromosome 3 and 8q aberrations had a much larger influence on survival in patients with a light iris compared to those with a brown iris. This suggests a synergistic effect of iris pigmentation and chromosome status in the regulation of oncogenic behavior of UM. Iris color should be taken into consideration when calculating the risk for developing metastases.

Introduction

Uveal melanoma (UM) is a rare but often fatal disease; around 50% of the patients develop metastases, despite modern treatment options [1]. The incidence of UM varies from 5.3-10.9 individuals per million in Caucasians, and is much lower in people with Asian or African ancestry [2]. A UM arises from melanocytes in the iris, ciliary body, or choroid, with the latter being the most common anatomic location [3].

It is believed that a uveal melanocyte can transform into a pre-malignant cell [1,4], and subsequently develop into a UM. A mutation in (usually) the *GNAQ* or *GNA11* gene is considered the first step in developing malignancy, as these have already been detected in choroidal nevi [5-7]. Secondary mutations often occur in the *BAP1*, *SF3B1,* or *EIF1AX* genes [8-11] and are of prognostic significance.

The tumorigenic pathways in UM are not yet fully understood. One factor that predisposes to develop a UM is a light iris color. Several studies have shown that people with gray or blue irises have a higher chance of developing a UM than individuals with brown irises [12-17]. However, few studies have focussed on the different characteristics of UM in patients with light and dark irises.

Iris color is genetically determined, and a region around the HERC2/OCA2 genes (located on chromosome 15) accounts for 74% of human iris color [18,19]. Genetic studies typically define three phenotypic categories of iris color: blue, intermediate, and brown, where the intermediate category represents green, hazel, and yellow-brown [20-22]. Both the quantity and type of melanin in uveal melanocytes determine iris color, with ocular melanocytes producing two types of melanin: eumelanin (dark brown and black) and pheomelanin (yellow, red, and light brown) [23]. It is thought that eumelanin is photoprotective for pigmented tissues while pheomelanin is phototoxic, capable of inducing DNA damage [24]. The presence and degree of eumelanin and pheomelanin vary similarly between skin and hair, contributing to various phenotypes. With regard to the eye, the pheomelanin/ eumelanin ratio leads to the three iris color groups [25,26].

Since the types of ocular melanin have such different biological characteristics, we wondered whether iris color is related to known prognostic parameters in UM, especially chromosome changes, and to survival. We therefore set out to determine whether these characteristics differ between UM patients divided into three groups: those with gray/ blue-colored eyes, those with green/hazel eyes and those with brown eyes.

Materials and Methods

Study approval

This research was approved by the Biobank of the Leiden University Medical Center (LUMC) (number: Uveamelanoomlab-2019-7, approval May 2019) and the Medical Ethics Committee (METC-number: B20.022). The research adhered to Dutch law and the tenets of the Declaration of Helsinki (World Medical Association of Declaration 1964; ethical principles for medical research involving human subjects).

Patient population LUMC cohort

We performed a retrospective cohort study of patients at the Department of Ophthalmology, LUMC, Leiden, The Netherlands and analysed all 412 UM patients, aged 8 to 92 years, who had been primarily enucleated for UM at the LUMC between 1993 and 2019, and for whom the iris color was known. We compared clinical, histopathological, and genetic data between three groups of patients with different iris colors (Table 1).

Table 1. Clinical, histopathological, and chromosome characteristics of 412 UM eyes with either a blue iris color, a green iris color, or a brown iris color.

CHARACTERISTIC	Cases *n* = 412	Blue iris *n* = 269, (%)*	Green iris	Brown iris	
			n = 79, (%)*	*n* = 64, (%)*	P
Gender	412				0.78[§]
Male		158 (59%)	43 (54%)	38 (59%)	
Female		111 (41%)	36 (46%)	26 (41%)	
Side	412				0.65[§]
OD		139 (52%)	43 (54%)	37 (58%)	
OS		130 (48%)	36 (46%)	27 (42%)	
Age at enucleation, mean, years (±SD)	412	64.3 (13.4)	58.8 (13.8)	62.9 (14.3)	0.002[‡]
Largest basal tumor diameter in mm, mean (±SD)	393	11.6 ±3.9	11.4 ±3.3	12.6 ± 4.5	0.17[‡]
Tumor thickness in mm, mean (±SD)	393	6.2 ±3.5	6.9 ±3.5	7.6 ±3.5	0.013[‡]
Ciliary body involvement	411				0.34[§]
No		128 (48%)	44 (56%)	34 (53%)	
Yes		141 (52%)	34 (44%)	30 (47%)	
AJCC stage (8^{th})					0.20[†]
I		40 (16%)	10 (14%)	7 (11%)	
IIA		77 (30%)	20 (27%)	13 (21%)	
IIB		61 (24%)	20 (27%)	24 (39%)	
IIIA, IIIB, IIIC		76 (30%)	24 (32%)	18 (29%)	

CHARACTERISTIC	Cases *n* = 412	Blue iris *n* = 269, (%)*	Green iris *n* = 79, (%)*	Brown iris *n* = 64, (%)*	P
Tumor Pigmentation	396				
No pigmentation		10 (4%)	2 (3%)	2 (3%)	<0.0001‡
Light pigmentation		126 (49%)	44 (58%)	15 (24%)	
Moderate pigmentation		77 (30%)	19 (25%)	22 (36%)	
Heavy pigmentation		45 (17%)	11 (15%)	23 (37%)	
Cell Type	411				0.40§
Spindle		62 (23%)	21 (27%)	11 (17%)	
Mixed + Epithelioid		207 (77%)	57 (73%)	53 (83%)	
Chromosome 3	324				0.45§
Disomy		93 (45%)	34 (53%)	22 (43%)	
Monosomy		116 (56%)	30 (47%)	29 (57%)	
Chromosome 8q	294				0.74§
No gain of 8q		94 (50%)	32 (52%)	19 (44%)	
Gain of 8q		95 (50%)	30 (48%)	24 (56%)	
BAP1 staining	153				0.37§
Positive		37 (40%)	18 (51%)	9 (35%)	
Negative		55 (60%)	17 (49%)	17 (65%)	
Vital status	412				0.27§
Alive		148 (55%)	54 (68%)	34 (53%)	
Death due to UM		87 (32%)	19 (24%)	23 (36%)	
Death due to other causes		34 (13%)	6 (8%)	7 (11%)	

* Percentages are rounded and may not total 100.
† Linear-by-Linear association
‡ Kruskal-Wallis test (KWT)
§ Fisher's Exact test (Fisher)
Data from Leiden University Medical Center (LUMC), Leiden, The Netherlands

Following routine procedures, after enucleation and the cutting of the globe, a sample of fresh tumor material was tested for chromosomal aberrations. Chromosome status was determined by either karyotyping, fluorescence in situ hybridization (FISH) or single-nucleotide polymorphism (SNP) assay as previously described [27]. A tumor was classified with monosomy of chromosome 3 (M3) if any test revealed monosomy 3. Chromosome 8q status was determined by either karyotyping or a SNP assay. If any of these two tests revealed a gain of 8q, this was noted as a gain. The eyes underwent conventional histopathological evaluation by a pathologist specialised in ophthalmic pathology. This evaluation included a macroscopic description of tumor pigmentation at grossing (categorized as: none, light, moderate, heavy). BAP1 status was obtained through immunohistochemistry (IHC) as previously described [28,29], and scored by an ophthalmic pathologist.

Tumor, lymph node, and metastases (TNM) staging was performed according to the 8th edition of the American Joint Committee on Cancer (AJCC) staging manual [30].

Follow-up time was defined as the time period between the date of enucleation and the moment of death or the last recorded follow-up. Data were updated in November 2019. Of the 412 patients, 129 (31%) had died from metastases, 47 (11%) from other causes, and 236 (57%) were still alive at the end of follow-up.

Data selection criteria and defining iris color groups

Out of 1216 patients that were part of the LUMC enucleation database, we had data on iris color of 412 patients, who were subsequently included in this study. Iris color was obtained from medical charts and clinical photographs, or if those were not informative, from the self-reported iris color, as retrieved from questionnaires that were filled out by patients as part of regular care. Eyes with a predominantly blue or gray iris were noted as blue eyes, eyes with a clearly brown iris were noted as a brown eye and the eyes with a green, hazel or a combination of green, light brown, hazel, and hints of blue were noted as green eyes.

Wills Eye Hospital cohort

A second cohort consisted of 1001 cases from Wills Eye Hospital (WHE), Philadelphia, United States, of whom we analyzed the cases indicated as being white (965 cases) [31]. We excluded iris tumors. Iris color was known for 934 cases (SupplementalTable 1). Of these, 527 (56%) had blue eyes, 87 (9%) green eyes, and 320 (34%) brown eyes. The chromosome status was known for all cases. Group A had disomy 3, normal 8q (n= 456), group B disomy 3, extra 8q (n= 124), group C monosomy 3, up to three copies of 8q (n= 243), and group D monosomy 3 and more than three copies of 8q (n= 111). Treatment consisted of enucleation in 57 (6%) patients, eye-preserving treatment in 874 (93.5%), observation in one case. At the time of evaluation, 918 (98%) were alive, 14 (1%) had died from UM, and two from other causes. A total of 127 cases had developed metastases.

Immunohistochemistry and mRNA analysis

Immune infiltrate was determined using immunofluorescence with antibodies against CD68 for macrophages (pixels/mm2) [32]. mRNA gene expression was determined using the Illumina HT-12 v4 chip (Illumina, San Diego, CA, USA) after mRNA isolation with an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) [33].

Statistical analysis

Clinical, histopathological, and cytogenetic data were collected in an SPSS datafile (IBM SPSS Statistics for Windows, Version 23.0; IBM Corp., Armonk, NY, USA). Statistical testing was subsequently performed in SPSS. Population characteristics were described using means and percentages. A Pearson $\chi 2$ test was used to analyse two groups of categorical data, and a Linear-by-Linear Association test was used in case of more than 2 categories. A Mann-Whitney U (MWU) test was used to compare two groups of numerical data with a non-parametrical distribution, a Kruskal-Wallis test was performed in case of more than two groups of numerical data with a non-parametric distribution or unequal sample size. Kaplan-Meier (KM) curves were made and Log Rank tests were used to test differences. A p value<0.05 was considered statistically significant.

Results

Patient characteristics

We first set out to compare patient and tumor characteristics between cases of UM who had previously been treated by enucleation at the LUMC in Leiden. Three groups of iris color were defined, based on differences in quantity and ratio of eumelanin and pheomelanin as described by Wakamatsu (Wakamatsu, Hu et al. 2008): grey/blue irises, green/hazel irises, and brown irises. Out of a total of 1216 patients, eye color was known for 412 cases: one group consisted of 269 patients (65%) with a gray or blue iris (thereafter referred to as blue iris), the second group of 79 cases (19%) with a green or hazel iris (thereafter referred to as green iris), and the third group of 64 cases (15.5%) with a brown iris (Table 1). When comparing the three groups, the patients in the green group were significantly younger than those with blue and brown eyes (with a mean of 58.8 versus 64.3 and 62.9 years, respectively, p = 0.002).

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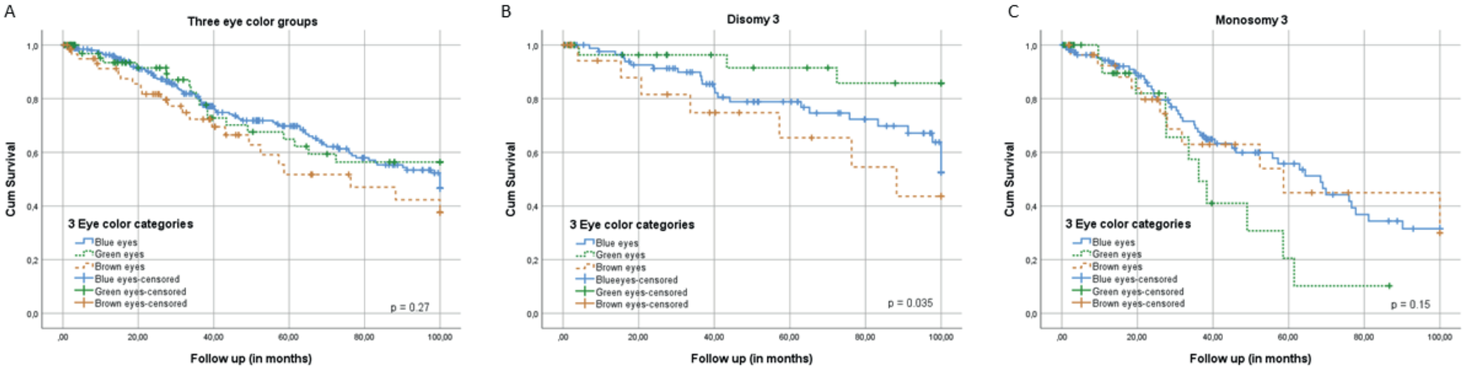
Iris pigmentation is related to tumor pigmentation

When comparing iris color with histopathological characteristics, no significant difference in largest basal diameter between the three groups was observed. However, tumors in blue eyes presented with the lowest tumor thickness with a mean of 6.2 mm, versus 6.9 mm in green eyes and 7.6 mm in brown eyes (p = 0.013). Major differences were noticed with regard to tumor pigmentation, with histopathologically lighter-colored tumors occurring in the lighter eyes: 61% of the eyes with a green iris had none-to-light tumor pigmentation, versus 53% of tumors in eyes with a blue iris and only 27% in the brown-iris eyes (p < 0.001) (Table 1).



A

Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Blue	269	200	131	99	66	46
Green	79	46	28	24	19	16
Brown	64	45	25	14	10	9

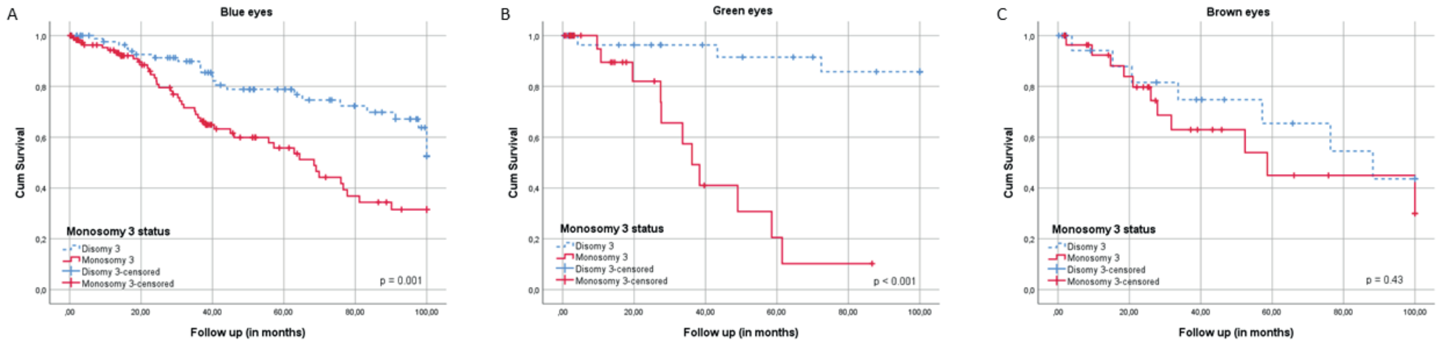
B

Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Blue	93	72	51	41	29	17
Green	34	24	20	18	15	14
Brown	22	14	10	7	5	4

C

Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Blue	116	73	40	26	15	10
Green	30	11	4	2	1	-
Brown	29	20	9	5	3	3

Figure 1. Melanoma-related survival in uveal melanoma patients divided over three iris color groups in the LUMC cohort (n = 412). (A) KM curves show the survival of patients with a UM and a blue iris (n=269), a green iris (n=79) or a brown iris (n=64). Survival between the three groups of iris color does not differ significantly (p = 0.27, Log Rank). When stratifying the tumors based on chromosome 3 status, the patients with different iris colors show a difference in survival when the tumor is D3 (B) (p = 0.035, Log Rank), but not when the tumor is M3 (C) (p = 0.15, Log Rank). Note that patients with green eyes have the best prognosis in D3 UM, but the worst in M3 UM. Abbreviations; D3 = Disomy 3, M3 = Monosomy 3.



Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Disomy 3	93	72	51	41	29	17
Monosomy 3	116	73	40	26	15	10

Time (months)	0	20	40	60	80	1-00
	Patients entering each interval					
Disomy 3	34	24	20	18	15	14
Monosomy 3	30	11	4	2	1	-

Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Disomy 3	22	14	10	7	5	4
Monosomy 3	29	20	9	5	3	3

Figure 2. Eye color influences the effect of chromosome 3 status on survival. (A) Survival in patients from the LUMC cohort with blue irises (n=209), categorized according to D3 (n=93) or M3 (n=116) status, differed significantly (p = 0.001, Log Rank). (B) Survival in patients with green irises (n=64), categorized according to D3 (n=34) or M3 (n=30) status, differed significantly (p < 0.001, Log Rank). (C) Survival in patients with brown irises (n = 51), categorized according to D3 (n=22) or M3 (n=29) status, did not differ significantly (p = 0.43, Log Rank). Abbreviations: D3 = Disomy 3, M3 = Monosomy 3.

8

Chromosome copy number changes and mutation status

Chromosome 3 and 8q status did not differ significantly between the three iris color groups (p=0.45 and p=0.74, respectively). Similarly, when looking at BAP1 staining of the UM (known for 153 cases), no significant difference was found between the three iris color groups (p = 0.37) (Table 1).

Survival of the overall LUMC cohort

As a previous study showed that light eye color is an important risk factor in UM, we analysed the effect of iris color on overall survival (Figure 1A), but no significant difference was observed (p = 0.27, Log Rank test).

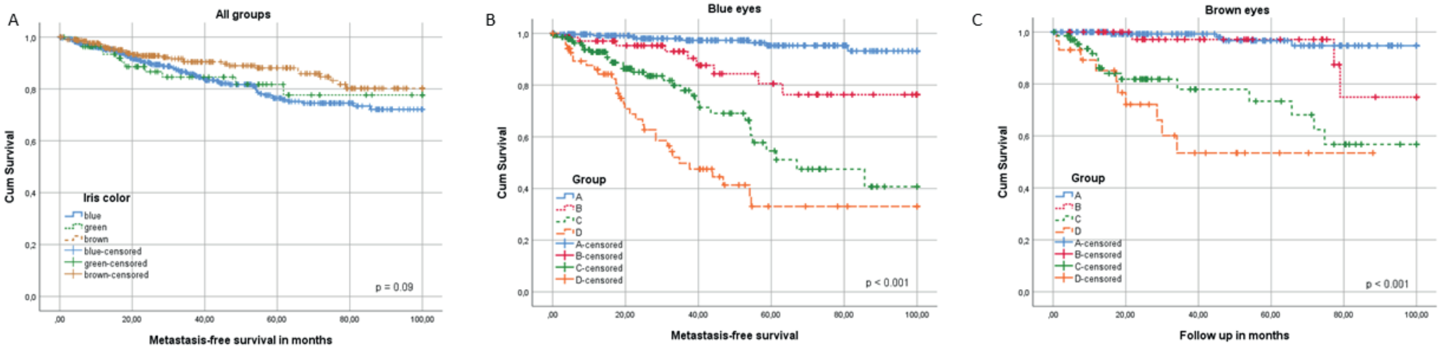
Survival for D3 and M3 tumors separately

We hypothesized that the potential effect of the presence of the phototoxic pheomelanin in light irises on survival might add to the effect of a tumor's chromosome status, and therefore compared the effect of iris color within groups with the same chromosome status. When looking within the D3 tumors (n = 149), the three iris color groups were found to differ in disease-related death (p = 0.035), with a brown iris color conferring the highest risk (Figure 1B). When looking at only the M3 tumors (n = 175), no significant differences between the iris color groups were observed (p = 0.15) (Figure 1C). When looking at these curves, we found it very striking that individuals with green iris had the BEST prognosis in D3 tumors, but the WORST in M3 tumors; we subsequently set out to compare the effect of chromosome copy number changes on survival within the three patient groups with different iris colors. Chromosome 3 status had a large impact on survival in patients with a green iris ($p < 0.0001$) and with a blue iris (p = 0.001), but not in those with a brown iris (p = 0.43) (Figure 2).

When looking at the influence of chromosome 8 copy number, the patients with blue/grey eyes or green eyes show the expected negative effect of a gain of 8q on survival, which is (again) not the case in the brown eye group (Supplemental Figure 1,).

Independent cohort

In order to validate our findings, we evaluated a second cohort (WEH) consisting of 934 cases of whom iris color and tumor chromosome status were known. The majority of cases in this cohort consisted of patients who had undergone an eye-preserving treatment (93.5%) (Supplemental Table 1). Compared to the LUMC cohort, this group was younger, tumors were thinner, with less frequent involvement of the ciliary body, and less often M3. As only a few patients died during follow-up, we took the date of development of UM metastases as end point for survival curves.



Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Blue	527	345	218	131	69	32
Green	87	52	34	21	14	10
Brown	319	210	133	90	48	21

Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Group A	246	182	132	87	48	22
Group B	70	55	32	21	10	5
Group C	142	73	33	17	7	2
Group D	69	35	21	6	4	3

Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Group A	171	122	84	56	32	13
Group B	50	36	25	16	6	5
Group C	69	35	17	15	9	3
Group D	29	17	7	3	1	-

Figure 3. Melanoma-related survival in uveal melanoma patients divided over three iris color groups in the WEH cohort (n = 933). (A) KM curves showing the survival of patients with a UM and a blue iris (n= 527), a green iris (n= 87) or a brown iris (n= 319). Survival between the three groups of iris color does not differ significantly (p = 0.09, Log Rank). (B) Survival in patients with a blue iris (n= 527), categorized according to group A (n= 246), group B (n= 70), group C (n= 142) or group D (n=69), differed significantly (p < 0.001, Log Rank). (C) Survival in patients with a brown iris (n = 319), categorized according to group A (n= 171), group B (n= 50), group C (n= 69) or group D (n= 29), differed significantly (p < 0.001, Log Rank). In the brown iris group, there was no significant different between group A and group B and between group C and group D.

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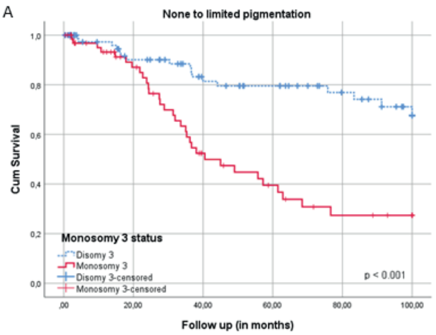
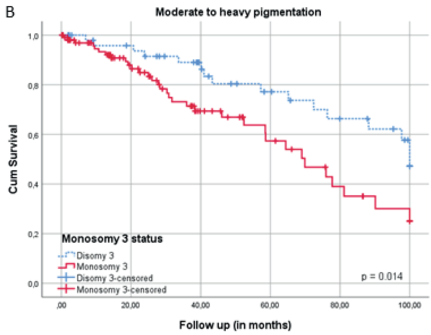
A



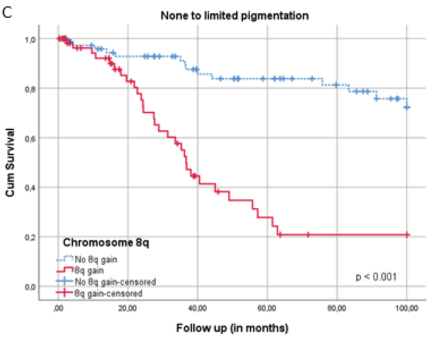
Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Disomy 3	88	61	44	38	28	20
Monosomy 3	70	42	21	14	8	6

B



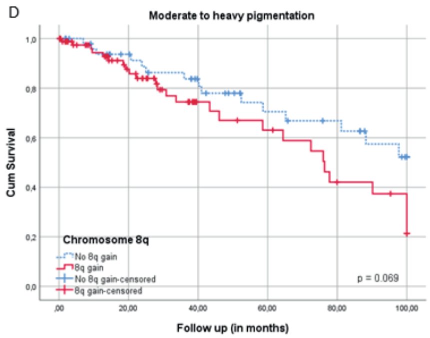
Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Disomy 3	54	44	32	23	17	11
Monosomy 3	103	60	31	18	10	6

C



Time (months)	0	20	40	60	80	100
	Patients entering each interval					
No 8q gain	86	61	46	39	31	22
8q gain	63	34	14	8	4	4

D



Time (months)	0	20	40	60	80	100
	Patients entering each interval					
No 8q gain	54	39	29	19	16	8
8q gain	85	48	20	16	9	7

Figure 4. Chromosome status influence survival dependent on the degree of tumor pigmentation (LUMC cohort). The influence of chromosome status on survival in lightly-pigmented tumors (D3 n=88, M3 n=70) (A), p < 0.001 (Log Rank) is higher than in highly-pigmented tumors (D3 n=54, M3 n=103)(B), p = 0.014 (Log Rank). Chromosome 8q status has a major influence on survival in lightly-pigmented tumors (8q n = 86, 8q abn n = 63) (C) (p < 0.001, Log Rank), and a minor role in darkly-pigmented tumors, not reaching a significant level (8q n=54, 8q abn n=85) (D) (p = 0.069, Log Rank). Abbreviations: D3 = Disomy 3, M3 = Monosomy 3.

When comparing the three iris color groups, we noticed that patients with a brown iris were significantly younger (Supplemental Table 1), and their tumors were less often M3 than tumors in patients with a blue iris (32% versus 40%, p = 0.002). The three curves for the development of metastases did not differ significantly between the three eye colors (Figure 3A). Combining information on chromosome 3 and 8q status according to four groups showed a large differential effect of eye color (Figure 3B, C). In brown eyes, the curves of groups A and B were not significantly different, and neither were the curves of groups C and D.

Tumor pigmentation and chromosome status

As we had noticed in the LUMC cohort that histological tumor pigmentation was highly correlated with iris color, we additionally analysed the influence of chromosome 3 and 8q status on survival in patients with either lightly-pigmented tumors or with highly-pigmented tumors. While chromosome 3 status affected survival in both groups, its influence was much larger in patients with lightly-pigmented tumors than in those with dark tumors ($p < 0.001$ and $p = 0.014$, respectively, Log Rank) (Figure 4A and B). The influence of chromosome 8q status on survival was overwhelming in lightly-pigmented tumors ($p < 0.001$, Log Rank), but minor in dark tumors, not reaching significance ($p = 0.069$, Log Rank)(Figure 4C and D). We did not have the histopathological tumor color of the validation group as the majority of these cases underwent an eye-sparing treatment.

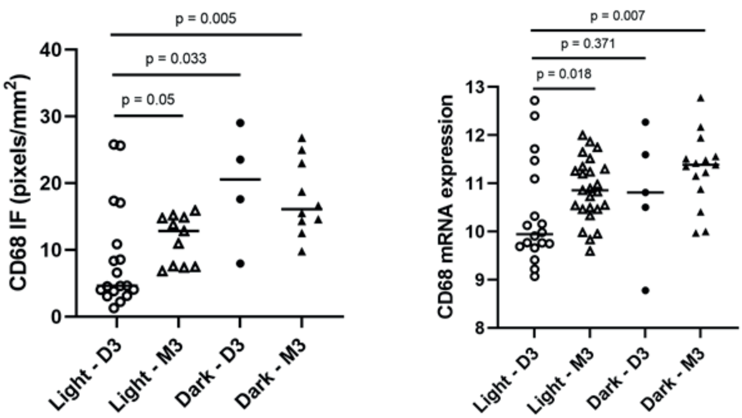


Figure 5. Both mRNA CD68 expression (A) and IF analysis of CD68 (B) show that inflammation is higher in M3 tumors than D3 tumors. However, this is more pronounced in tumors with none to limited pigmentation than in tumors with heavy pigmentation. The darker tumors showed a high level of inflammation, independent of chromosome 3 status. mRNA expression was available in 64 cases, IF analysis was available in 43 cases. P-values were calculated with Mann-Whitney U tests.

Inflammation and tumor pigmentation

We wondered whether the differences in survival could be explained by the amount of inflammation, as we had previously observed that prognosis in UM is related to the presence of an inflammatory phenotype and that this is related to chromosome 3 status [34]. We would expect more inflammation in M3 tumors, especially the lightly-pigmented ones. One characteristic of inflammation is the presence of macrophages.

Makitie (2001) showed that the number of CD68+ macrophages correlated with a high microvascular density and a high 10-year mortality [35]. Therefore, we compared the inflammatory marker CD68 between four groups of tumors (based on light or dark tumor pigmentation, and D3 or M3). We studied the immunohistochemical staining for CD68+ macrophages as well as at mRNA expression of CD68 in the analysed samples. While we confirmed that loss of one chromosome 3 is related to a higher macrophage infiltrate, this relation was especially seen in lightly-pigmented tumors, while in tumors with heavy pigmentation, inflammation was high, independent of chromosome 3 status (Figure 5). We did not have sufficient data on eye color to study the relationship between iris color and inflammation.

Discussion

When we analysed the characteristics of UM from eyes with different iris colors, we observed that chromosome copy changes had a much greater influence on survival when the UM occurred in an eye with a light (blue/grey and hazel/green) iris than when the eye had a dark (brown) iris. In both the LUMC and WEH cohort, this was observed for loss of chromosome 3 as well as for gain of copies of chromosome 8q.

Iris color is an important predisposing factor in the development of UM. This was already recognized in the 1980s [36], and later confirmed in larger population-based studies [12,15,37-39]. People from African or Asian heritage (with darker iris colors) have a very low incidence of UM, and we see a South-to-North increase of UM in Europe, which corresponds to increasing incidences of light iris color [40]. Seddon showed that in the US, a northern European heritage is one of the risk factors of developing UM [41]. Ferguson has studied 28 SNPs, previously identified as risk variants in genome-wide association studies on UM and cutaneous melanoma, and found that the three most important variants were located on 15q12 in the region of *HERC2/OCA2*. These findings imply a strong role for pigmentation-related genes on the risk of developing a UM [42].

While the relation between iris color and UM has been well-reported, only a few studies looked at the relation between iris color and survival in these patients. One study in Europe, which investigated the relation between iris color and UM-related death in 459 cases, reported that a light iris color was associated with a worse survival [17]. In the US, Regan analysed a series of 1162 patients who had been treated with proton beam irradiation for UM [43]. In Regan's study, patients with blue/gray irises also showed a worse survival than those with dark irises.

In the current study, we do not see a direct correlation between iris color and prognosis in any of the two cohorts. We analysed the presence of chromosome 3 abnormalities versus eye color and did not see a difference between blue and brown eyes in the LUMC cohort, while there were slightly less M3 tumors among the brown eyes in the WEH cohort (40% in blue eyes, 32% in brown eyes). Regan mentioned that tumor pigmentation was related to iris color, which finding we repeat here in the LUMC cohort. Regan found that a high tumor pigmentation was related to a worse survival. Several other studies analyzed the relation between tumor pigmentation and metastasis formation, and similarly observed a lower risk of developing metastases in amelanocytic lesions [44]. Rothermel observed that the degree of pigmentation of the UM influenced the immunogenicity, with hypopigmented UM leading to a higher anti-tumor T-cell reactivity in metastatic disease [45].

When we compared the effect of copy number changes within the eye color groups in the LUMC cohort, we noticed striking differences in the effect of M3: M3 is known to be associated with the development of metastases, but we only observed a differential effect of loss of one chromosome 3 in the groups of blue and green eyes, and not in the group with brown eyes. Although the WEH group had a lower number of high-risk cases, and we used the development of metastases instead of death due to metastases as endpoint, we similarly noticed that chromosome aberrations were a better prognosticator in blue-eyed patients than in brown-eyed patients.

We propose that the presence of pheomelanin enhances the effect of genetic aberrations, such as M3 and 8q gain. A similar observation has been made in mice with regard to BRAF mutations: Mitra [46] observed that mice with an abundance of pheomelanin and therefore red fur responded differently to a BRAF (V600E) mutation than mice with eumelanin: a conditional, melanocyte-targeted BRAF mutation introduced in mice with abundant eumelanin (and consequently black fur) induced only sporadic cutaneous melanoma. When the MC1R was mutated, leading to a shift to pheomelanin and the presence of a red fur, about 50% of the mice with the BRAF mutation developed cutaneous melanoma. This effect was ultraviolet-irradiation independent.

We similarly propose a role for the type of ocular pigmentation, with a synergistic effect occurring between pheomelanin and loss of chromosome 3 and/or gain of chromosome 8q, making the tumor more malignant.

The type of pigment may influence inflammation in the choroid and in the tumor. We know that blue and green eyes contain a higher ratio of pheomelanin versus eumelanin than brown eyes. Pheomelanin is considered a risk factor for the development of reactive oxygen species (ROS), and the development of inflammation, as already described for the skin [47]. Individuals with the red hair/fair skin phenotype, associated with pheomelanin, are known to show a high sensitivity to sunburn, and are prone to develop cutaneous melanoma [48]. Experimental work demonstrates that UV irradiation of a skin with pheomelanin leads to an increase in oxygen radicals, with a higher production of cytokines such as TNFalfa, IL1, and IL6, with an inflammatory cell influx with myeloid cells. These are especially myeloid-derived suppressor cells, while incoming macrophages show M2 polarisation, creating a pro-tumor environment [49]. The type of melanin determines the sensitivity to the induction of mutations, with a light skin being more sensitive. UV irradiation of skin high in eumelanin on the other hand does not induce this inflammatory response, does not lead to high numbers of mutations, and in addition, is associated with better specific immune responses, such as cytotoxic T cells, delivering an environment that acts against tumor development [50].

We previously described that M3/loss of BAP1 expression in UM is related to an inflammatory phenotype [34,51]. We observe that M3/loss of BAP1 especially influences the development of metastases in patients with a green or blue iris, two eye colors associated with a high pheomelanin/ eumelanin ratio. Our hypothesis states that the presence of pheomelanin and possible exposure to light helps to trigger inflammation in a UM that is confronted with M3. This is supported by a study on malignant mesothelioma: mice with a germline *BAP1* mutation (BAP1$^{+/-}$) exposed to low levels of asbestos that otherwise rarely induce malignant mesothelioma (MM) in WT mice, showed significant levels of inflammation in BAP1$^{+/-}$ mice [52]. Significantly higher levels of M2 macrophages were observed in the BAP1$^{+/-}$ mice. We hypothesize that other triggers such as sunlight may similarly stimulate inflammation in pre-disposed eyes, i.e. those with relatively more pheomelanin compared to eumelanin.

A possible limitation is that iris color is a clearly visible feature, however, interpretation of iris color is subjective and different classification schemes are used: e.g., one study describes 24 different iris colors in detail [53], while others used two groups of iris colors [43]. We split the group into three iris colors on the basis of the biological differences in the distribution of eumelanin and pheomelanin [26,54].

In conclusion, chromosome aberrations had more impact when the patient had a light iris than when patient had a brown iris. Gaining knowledge on the pigmentation pathways in the pathogenesis in UM might lead to a better understanding of the development of metastases. We suggest that iris color should be taken into consideration when advising patients regarding the risk for developing metastases, using more hesitancy when advising dark-eyed patients .

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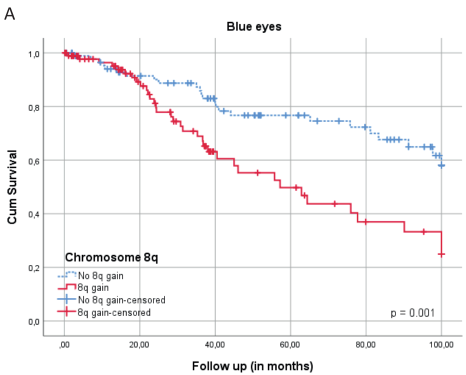
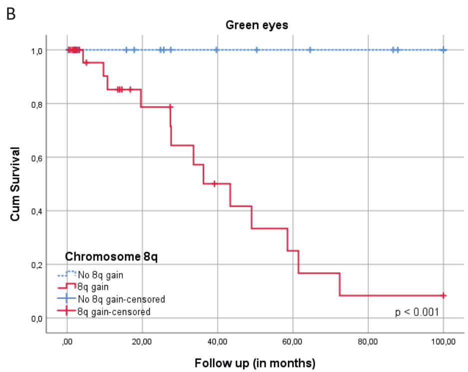
Supplemental Table S1. Clinical and chromosome characteristics of 934 UM eyes (verification group) based on three iris colors.

CHARACTERISTIC	Cases *n* = 934	Blue iris *n* =527, (%)*	Green iris *n* =87, (%)*	Brown iris *n* =320, (%)*	P
Gender	934				0.69[§]
Male		280 (53%)	43 (49%)	162 (51%)	
Female		247 (47%)	44 (51%)	158 (49%)	
Side	934				0.62[§]
OD		270 (51%)	49 (56%)	171 (53%)	
OS		257 (49%)	38 (44%)	149 (47%)	
Age at diagnosis, mean, years (±SD)	934	59.4 (±13.6)	58.5 (±13.8)	56.6 (±14.1)	0.006[‡]
Largest basal tumor diameter in mm, mean (±SD)	934	12.4 (±4.3)	11.9 (±4.3)	12.0 (±4.3)	0.41[‡]
Tumor thickness in mm, mean (±SD)	934	5.8 (±3.3)	5.4 (±3.5)	5.5 (3.0)	0.45[‡]
Ciliary body involvement	934				0.58[§]
No		481 (91%)	81 (93%)	298 (93%)	
Yes		46 (9%)	6 (7%)	22 (7%)	
AJCC T category	934				0.40[§]
1		191 (36%)	34 (39%)	115 (36%)	
2		126 (24%)	22 (25%)	98 (31%)	
3		157 (30%)	24 (28%)	78 (24%)	
4		53 (10%)	7 (8%)	29 (9%)	
Chromosome 3	934				0.002[§]
Disomy		315 (60%)	43 (49%)	219 (68%)	
Monosomy		212 (40%)	44 (51%)	101 (32%)	
Chromosome 8q	932				0.26[§]
No gain of 8q		274 (52%)	44 (51%)	184 (57%)	
Gain of 8q		251 (48%)	43 (49%)	136 (42%)	
Vital status	934				0.11[§]
No metastasis		446 (85%)	74 (85%)	287 (90%)	
Metastasis		81 (15%)	13 (15%)	33 (10%)	

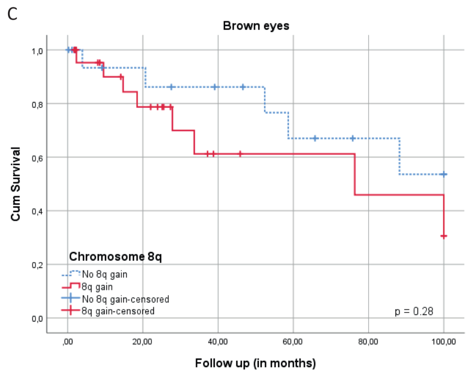
* Percentages are rounded and may not total 100.
[§] Pearson's chi square test
‡ Kruskal-Wallis test (KWT)
Data from the Ocular Oncology Service, Wills Eye Hospital (WEH), Philadelphia, United States.



Time (months)	0	20	40	60	80	100
	Patients entering each interval					
No 8q gain	94	69	52	39	31	17
8q gain	95	57	24	18	10	8



Time (months)	0	20	40	60	80	100
	Patients entering each interval					
No 8q gain	32	22	17	16	15	13
8q gain	30	12	6	3	1	1



Time (months)	0	20	40	60	80	100
	Patients entering each interval					
No 8q gain	19	13	10	7	5	4
8q gain	24	14	5	4	3	3

Supplemental Figure 1. Eye color influences the effect of chromosome 8q status on survival (LUMC cohort). (A) Survival in patients from the LUMC cohort with a blue iris (n=189), categorized according to 8q normal (n= 94) or 8q gain (n=95) status, differed significantly (p = 0.001, Log Rank). (B) Survival in patients with a green iris (n=62), categorized according to 8q normal (n= 32) or 8q gain (n= 30), differed significantly (p < 0.001, Log Rank). (C) Survival in patients with a brown iris (n = 43), categorized according to 8q normal (n= 19) or 8q gain (n= 24) status, did not differ significantly (p = 0.28, Log Rank).

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