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

Design: We determined iris color in a large cohort of patients enucleated for UM. Clinical and histopathologic tumor characteristics, chromosome status, and survival were compared among 3 groups on the basis of iris color.

Participants: A total of 412 patients with choroidal/ciliary body UM, who had undergone primary enucleation at the Leiden University Medical Center, Leiden, The Netherlands, between 1993 and 2019, were divided into 3 groups based on iris color: gray/blue, green/hazel, and brown. The validation cohort included 934 patients with choroidal/ciliary body UM treated at Wills Eye Hospital (WEH).

Methods: 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 3 iris color groups, in relation to the tumor's chromosome 3 and 8q status.

Results: Moderate and heavy tumor pigmentations were especially seen in eyes with a brown iris ($P < 0.001$). Survival did not differ between patients with different iris colors ($P = 0.27$); however, in 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. Likewise, 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: Although iris color by itself did not relate to UM-related survival, chromosome 3 and 8q aberrations had a larger influence on survival in patients with a light iris than 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 a patient's risk for developing metastases. *Ophthalmology 2022;129:421-430 © 2021 by the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).*

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Uveal melanoma (UM) is a rare but often fatal disease; approximately 50% of patients with UM develop metastases despite modern treatment options.[1] The incidence of UM varies from 5.3 to 10.9 individuals per million in Whites and is lower in people of Asian or African ancestry.[2] Uveal melanoma 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 premalignant 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 because these have already been detected in choroidal nevi.[5-7] Secondary mutations often occur in the *BAP1*, *SF3B1* (*Splicing Factor 3b Subunit 1),* or *EIF1AX* (Eukaryotic Translation Initiation Factor 1A X-Linked) 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

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with gray or blue irises have a higher chance of developing a UM than individuals with brown irises[12-17]; however, few studies have focused 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* (HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase 2/OCA2 Melanosomal Transmembrane Protein) genes (located on chromosome 15) accounts for 74% of human iris color.[18,19] Genetic studies typically define 3 phenotypic categories of iris color: blue, intermediate, and brown; the intermediate category represents green, hazel, and yellow-brown.[20-22] Both the quantity and the type of melanin in uveal melanocytes determine iris color, with ocular melanocytes producing 2 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, whereas 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 3 iris color groups.[25,26]

Because 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 set out to determine whether these characteristics differ between UM patients divided into 3 groups: those with gray/blue-colored eyes, those with green/hazel eyes, and those with brown eyes.

Methods

Study Approval

This research was approved by the Biobank of the Leiden University Medical Center (LUMC) (no.: Uveamelanoomlab-2019-7, approval May 2019) and the Medical Ethics Committee (no.: 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). All participants provided informed consent.

Patient Population in the LUMC Cohort

We performed a retrospective cohort study of patients at the Department of Ophthalmology, LUMC, Leiden, The Netherlands, and analyzed 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, histopathologic, and genetic data among 3 groups of patients with different iris colors (Table 1).

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 karyotyping, fluorescence in situ hybridization, 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 karyotyping or SNP assay. If either of these 2 tests revealed a gain of 8q, this was noted as a gain. The eyes underwent conventional histopathologic evaluation by a pathologist specialized 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 as previously described[28,29] and scored by an ophthalmic pathologist.

Tumor, lymph node, and metastases staging was performed according to the 8th edition of the American Joint Committee on Cancer 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%) died as the result of metastases, 47 (11%) died of other causes, and 236 (57%) were still alive at the end of follow-up.

Data Selection Criteria and Defining Iris Color Groups

Of 1216 patients who were part of the LUMC enucleation database, we had data on the 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 brown eyes, 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 (WEH) Cohort

A second cohort consisted of 1001 cases from WEH, Philadelphia, PA, of whom we analyzed the cases indicated as being White (965 cases).[31] We excluded iris tumors. Iris color was known for 934 cases (Table S1, available at www.aaojournal.org). Of these, 527 (56%) had blue eyes, 87 (9%) had green eyes, and 320 (34%) had brown eyes. The chromosome status was known for all cases. Group A had disomy 3, normal 8q (n = 456); group B had disomy 3, extra 8q (n = 124); group C had monosomy 3, up to 3 copies of 8q (n = 243); and group D had monosomy 3 and more than 3 copies of 8q (n = 111). Treatment consisted of enucleation in 57 patients (6%), eye-preserving treatment in 874 patients (93.5%), and observation in 1 patient. At the time of evaluation, 918 patients (98%) were alive, 14 patients (1%) had died of UM, and 2 patients died of other causes. A total of 127 patients had developed metastases.

Immunohistochemistry and mRNA Analysis

Immune infiltrate was determined using immunofluorescence with antibodies against CD68 for macrophages (pixels/mm^2).[32] The mRNA gene expression was determined using the Illumina HT-12 v4 chip (Illumina) after mRNA isolation with an RNeasy Mini Kit (Qiagen).[33]

Statistical Analysis

Clinical, histopathologic, and cytogenetic data were collected in an SPSS data file (IBM SPSS Statistics for Windows, Version 23.0; IBM Corp). Statistical testing was subsequently performed in SPSS. Population characteristics were described using means and percentages. A Pearson chi-square test was used to analyze 2 groups of categorical data, and a linear-by-linear association test was used in the case of more than 2 categories. A Mann−Whitney *U* test was used to compare 2 groups of numerical data with a nonparametrical distribution. A Kruskal−Wallis test was performed in the case of more than 2 groups of numerical data with a nonparametric distribution or unequal sample size.

Table 1. Clinical, Histopathologic, and Chromosome Characteristics of 412 UM Eyes with a Blue Iris Color, Green Iris Color, or Brown Iris Color

Characteristic	Cases n = 412	Blue Iris n = 269, (%)*	Green Iris n = 79, (%)*	Brown Iris 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§
Right eye		139 (52)	43 (54)	37 (58)	
Left eye		130 (48)	36 (46)	27 (42)	
Age at enucleation, mean, yrs (± 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})	390				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)	
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)	

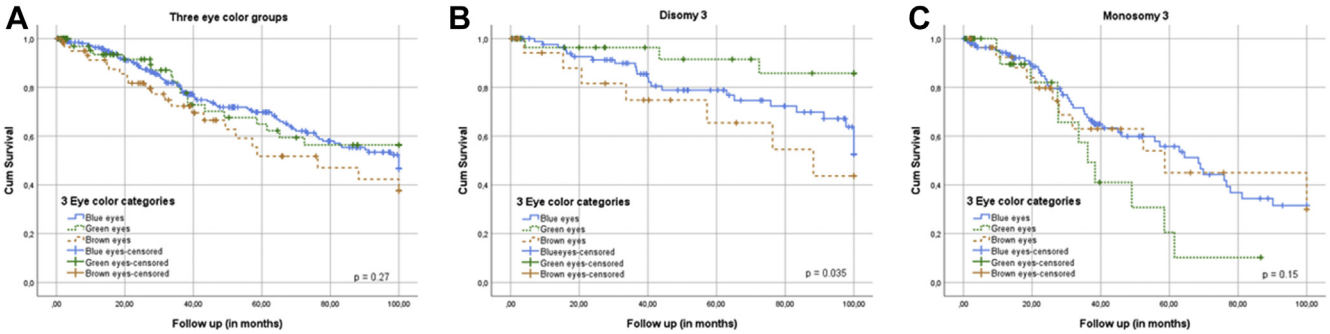
AJCC = American Joint Committee on Cancer; SD = standard deviation; UM = uveal melanoma.
Data from Leiden University Medical Center, Leiden, The Netherlands.
*Percentages are rounded and may not total 100.
†Linear-by-linear association.
‡Kruskal–Wallis test.
§Fisher exact test.

Kaplan–Meier 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 UM patients who had previously been treated by enucleation at the LUMC in Leiden. Three groups of iris color were defined on the basis of differences in quantity and ratio of eumelanin and pheomelanin as described by Wakamatsu et al[26]: gray/blue irises, green/hazel irises, and brown irises. Of a total of 1216 patients, eye color was known for 412: The first group consisted of 269 patients (65%) with a gray or blue iris (thereafter referred to as "blue iris"), the second group consisted of 79 patients (19%) with a green or hazel iris (thereafter referred to as "green iris"), and the third group consisted of 64 patients (15.5%) with a brown iris (Table 1). When comparing the 3 groups, the patients in the green group were significantly younger than those with blue and brown eyes (with a mean of 58.8 vs. 64.3 and 62.9 years, respectively, $P = 0.002$).



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

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

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 patients with uveal melanoma (UM) divided over 3 iris color groups in the Leiden University Medical Center cohort (n = 412). **A,** Kaplan–Meier curves show the survival of patients with a UM and a blue iris (n = 269), green iris (n = 79), or brown iris (n = 64). Survival among the 3 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. D3 = disomy 3; M3 = monosomy of chromosome 3.

Iris Pigmentation Is Related to Tumor Pigmentation

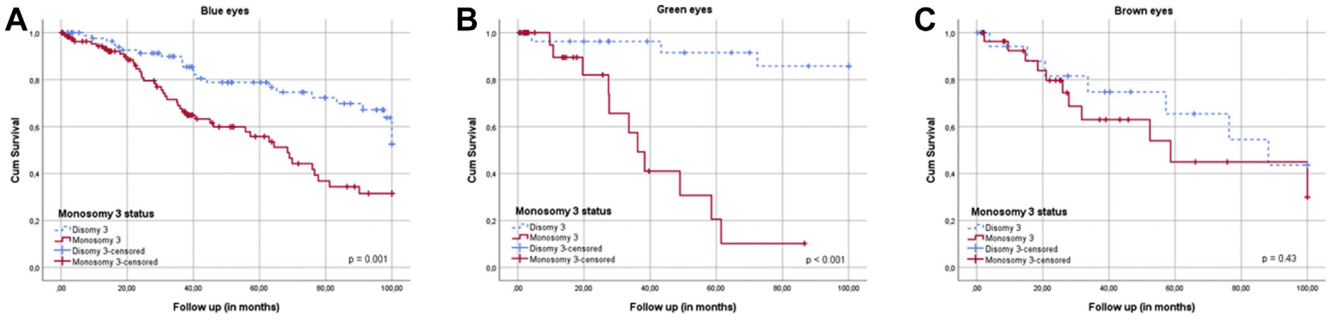
When comparing iris color with histopathologic characteristics, no significant difference in largest basal diameter among the 3 groups was observed; however, tumors in blue eyes presented with the lowest tumor thickness with a mean of 6.2 mm versus 6.9 mm in green eyes and 7.6 mm in brown eyes ($P = 0.013$). 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).

Chromosome Copy Number Changes and Mutation Status

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

Survival of the Overall LUMC Cohort

Because a previous study showed that light eye color is an important risk factor in UM,[34] we analyzed the effect of iris color

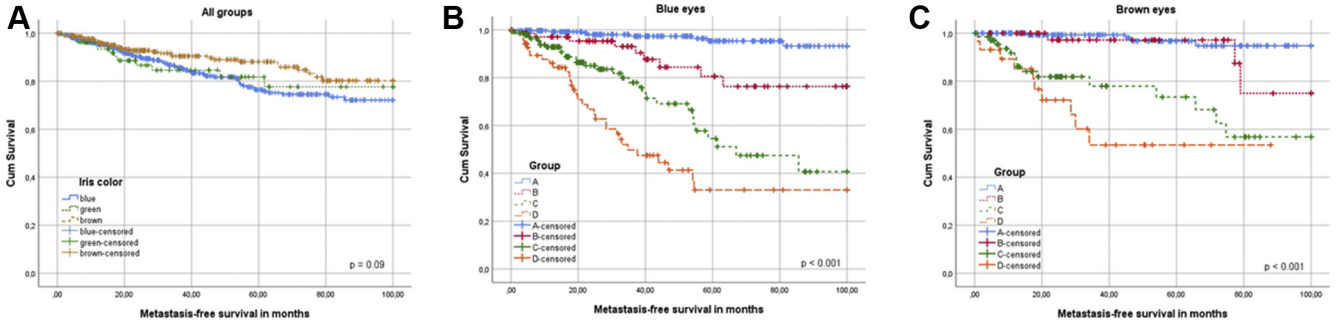


Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Disomy 3	93	72	51	41	29	17
Monosomy 3	116	73	40	26	15	10

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 Leiden University Medical Center 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). D3 = disomy 3; M3 = monosomy of chromosome 3.



Time (months)	0	20	40	60	80	100
	Patients entering each interval					
Blue/grey	527	345	218	131	69	32
Hazel/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 patients with uveal melanoma (UM) divided over 3 iris color groups in the Wills Eye Hospital cohort (n = 933). **A,** Kaplan–Meier curves showing the survival of patients with a UM and a blue iris (n = 527), green iris (n = 87), or brown iris (n = 319). Survival among the 3 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 groups A and B and between groups C and D.

on overall survival (Fig 1A), but no significant difference was observed ($P = 0.27$, log-rank test).

Survival for Disomy 3 (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 3 iris color groups were found to differ in disease-related death ($P = 0.035$), with a brown iris color conferring the highest risk (Fig 1B). When looking at only the M3 tumors (n = 175), no significant differences among the iris color groups were observed ($P = 0.15$) (Fig 1C). When looking at these curves, we found it striking that individuals with a 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 3 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 a blue iris ($P = 0.001$) but not on those with a brown iris ($P = 0.43$) (Fig 2).

When looking at the influence of chromosome 8 copy number, the patients with blue/gray eyes or green eyes showed the expected negative effect of a gain of 8q on survival, which was (again) not the case in the brown eye group (Fig S1, available at www.aaojournal.org).

Missing Information

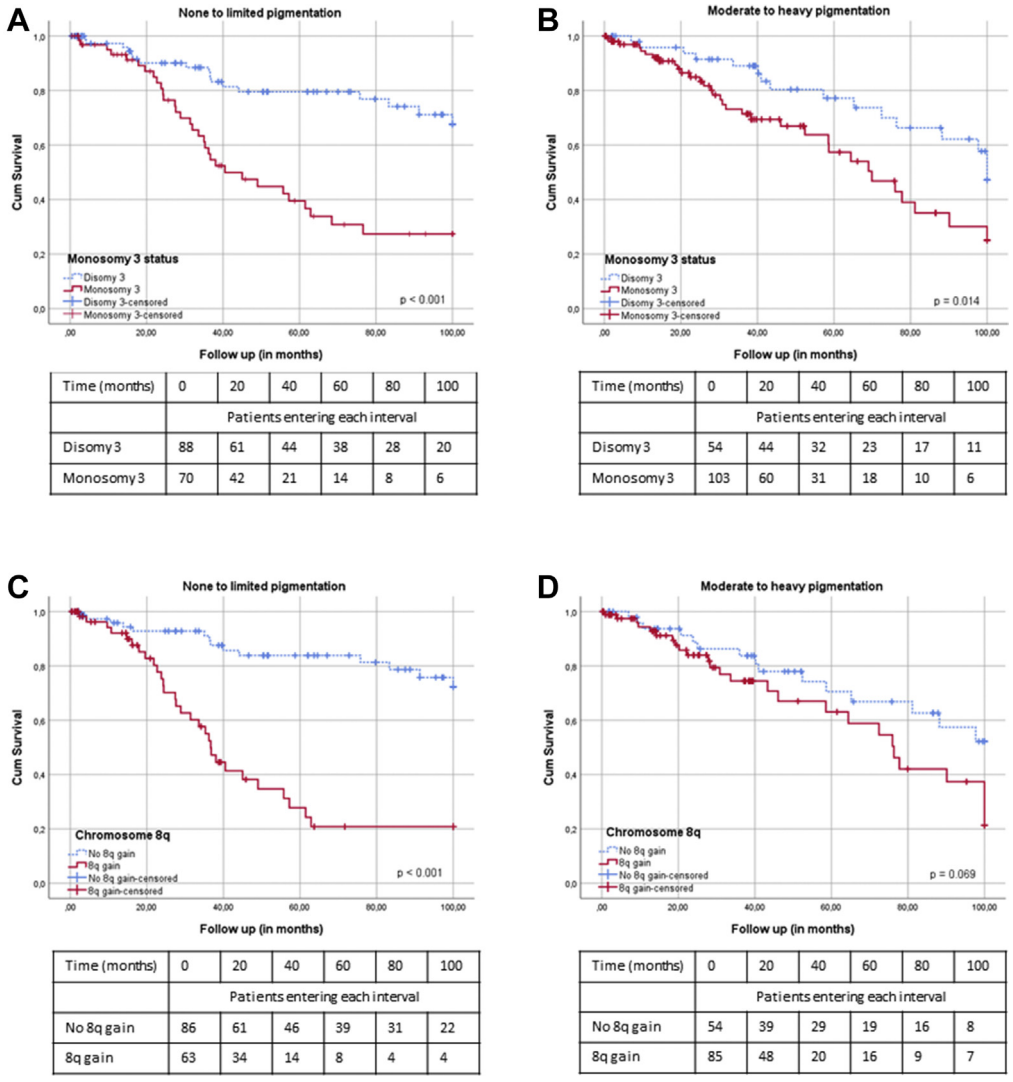
To address the fact that several cases had missing iris color or chromosome information, we performed some additional analyses. We found similar distribution of chromosome 3 and chromosome 8q aberrations in eyes with iris color information and without iris color information, as reported in Table S2A and B (available at www.aaojournal.org).

Moreover, we compared tumor features between cases with complete and missing chromosome 3 data, and the results are shown in Table S3 (available at www.aaojournal.org). We restricted the analysis on UM enucleated from 1999 onwards because none of the cases before 1999 were tested for chromosome status. The distribution of iris color and tumor pigmentation did not show a significant difference between the 2 groups ($P = 0.86$ and $P = 0.33$, respectively), nor did age at diagnosis ($P = 0.48$), ciliary body involvement ($P = 0.06$), and cell type ($P = 0.05$); however, UMs with known chromosome 3 status are significantly larger (higher largest basal diameter, $P = 0.002$; thickness, $P < 0.001$; American Joint Committee on Cancer stage, $P < 0.001$) than tumors with unknown chromosome 3 status.

Independent Cohort

To validate our findings, we evaluated a second cohort (WEH) consisting of 934 patients in 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%) (Table S1, available at www.aaojournal.org). Compared with the LUMC cohort, this group was younger, and tumors were thinner, with less frequent involvement of the ciliary body and less often M3. Because only a few patients died during follow-up, we took the date of development of UM metastases as the end point for survival curves.

When comparing the 3 iris color groups, we noticed that patients with a brown iris were significantly younger (Table S1, available at www.aaojournal.org) and their tumors were less often M3 than tumors in patients with a blue iris (32% vs. 40%, $P = 0.002$). The 3 curves for the development of metastases did not differ significantly among the 3 eye colors (Fig 3A). Combining information on chromosome 3 and 8q status according to 4 groups showed a large differential effect of eye color (Fig 3B, C). In brown eyes, the curves of groups A and B



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

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

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

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 influences survival depending 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, although not reaching significance (8q, n = 54; 8q abn, n = 85) **(D)** ($P = 0.069$, log rank). D3 = disomy 3; M3 = monosomy of chromosome 3.

were not significantly different, and neither were the curves of groups C and D.

Tumor Pigmentation and Chromosome Status

Because we noticed that histological tumor pigmentation was highly correlated with iris color in the LUMC cohort, we additionally analyzed the influence of chromosome 3 and 8q status on survival in patients with lightly pigmented tumors or highly pigmented tumors. Although chromosome 3 status affected survival in both groups, its influence was larger in patients with lightly pigmented tumors than in those with dark tumors ($P < 0.001$ and $P = 0.014$, respectively, log rank) (Fig 4A, 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) (Fig 5C, D). We did not have the histopathologic tumor color of the WEH validation group because the majority of these cases underwent an eye-sparing treatment.

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.[35] We would expect more inflammation in M3 tumors, especially the lightly pigmented ones. One characteristic of inflammation is the presence of macrophages. Makitie et al[36] showed that the number of CD68+ macrophages correlated with a high microvascular density and a high 10-year mortality. Therefore, we compared the inflammatory marker CD68 among 4 groups of tumors (based on light or dark tumor pigmentation and D3 or M3). We studied the immunohistochemical staining for CD68+ macrophages and mRNA

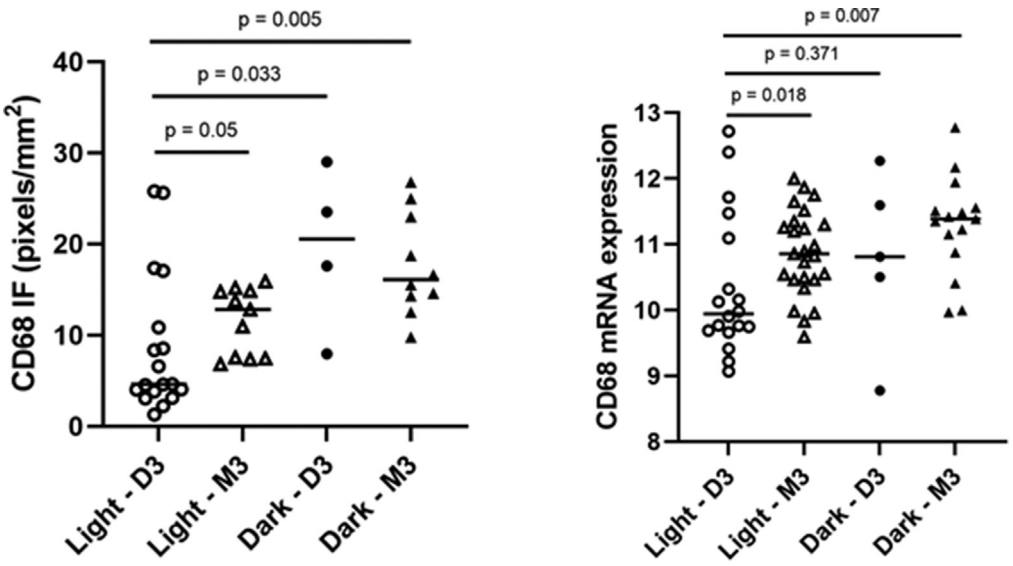


Figure 5. Both mRNA CD68 expression **(A)** and immunofluorescence analysis of CD68 **(B)** show that inflammation is higher in M3 tumors than D3 tumors; however, this is more pronounced in tumors with no to limited pigmentation than in tumors with heavy pigmentation. The darker tumors showed a high level of inflammation, independent of chromosome 3 status. The mRNA expression was available in 64 cases, and immunofluorescence analysis was available in 43 cases. *P* values were calculated with Mann−Whitney *U* tests. D3 = disomy 3; M3 = monosomy of chromosome 3; IF = immunofluorescence.

expression of CD68 in the analyzed samples. Although we confirmed that loss of one chromosome 3 is related to a higher macrophage infiltrate, this relation was especially seen in lightly pigmented tumors, whereas in tumors with heavy pigmentation, inflammation was high, independent of chromosome 3 status (Fig 5). We did not have sufficient data on eye color to study the relationship between iris color and inflammation.

Discussion

When we analyzed the characteristics of UM from eyes with different iris colors, we observed that chromosome copy changes had a greater influence on survival when the UM occurred in an eye with a light (blue/gray and hazel/green) iris than when the eye had a dark (brown) iris. In both the LUMC and WEH cohorts, this was observed for loss of chromosome 3 and 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[37] and later confirmed in larger population-based studies.[12,15,38–40] People from African or Asian heritage (with darker iris colors) have a 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.[41] Seddon et al[42] showed that in the United States, a northern European heritage is one of the risk factors of developing UM. Ferguson et al[43] studied 28 SNPs, previously identified as risk variants in genome-wide association studies on UM and cutaneous melanoma, and found that the 3 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.[43]

Although 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 United States, Regan et al[44] analyzed a series of 1162 patients who had been treated with proton beam irradiation for UM. In the study by Regan et al, 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 the 2 cohorts. We analyzed the presence of chromosome 3 abnormalities versus eye color and did not see a difference between blue and brown eyes in the LUMC cohort, whereas there were slightly less M3 tumors among the brown eyes in the WEH cohort (40% in blue eyes, 32% in brown eyes). Regan et al[44] mentioned that tumor pigmentation was related to iris color, which we also found in the LUMC cohort. Regan et al 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.[45] Rothermel et al[46] observed that the degree of pigmentation of the UM influenced the immunogenicity, with hypopigmented UM leading to a higher antitumor T-cell reactivity in metastatic disease.

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 the end point, 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 et al[47] 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, approximately 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 or gain of chromosome 8q, making the tumor more malignant.

The type of pigment may influence inflammation in the choroid and 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 and the development of inflammation, as already described for the skin.[48] 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.[49] Experimental work demonstrates that ultraviolet irradiation of a skin with pheomelanin leads to an increase in oxygen radicals, with a higher production of cytokines such as tumor necrosis factor alpha and interleukin 1 and 6, with an inflammatory cell influx with myeloid cells. These are especially myeloid-derived suppressor cells, whereas incoming macrophages show M2 polarization, creating a protumor environment.[50] The type of melanin determines the sensitivity to the induction of mutations, with a light skin being more sensitive. On the other hand, ultraviolet irradiation of skin high in eumelanin does not induce this inflammatory response, does not lead to high numbers of mutations, and is associated with better specific immune responses, such as cytotoxic T cells, delivering an environment that acts against tumor development.[51]

We previously described that M3/loss of BAP1 expression in UM is related to an inflammatory phenotype.[35,52] We observe that M3/loss of BAP1 especially influences the development of metastases in patients with a green or blue iris, 2 eye colors associated with a high pheomelanin/eumelanin ratio. Our hypothesis states that the presence of pheomelanin and possible exposure to light help 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 in wild-type mice showed significant levels of inflammation in $BAP1^{+/-}$ mice.[53] 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 predisposed eyes, that is, those with relatively more pheomelanin than 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: For example, 1 study describes 24 different iris colors in detail,[54] whereas others used 2 groups of iris colors.[44] We split the group into 3 iris colors on the basis of the biological differences in the distribution of eumelanin and pheomelanin.[26,55] One further limitation may be the missing data on chromosome status, and our analysis shows that UMs with known chromosome status are larger than those with unknown chromosome status. This is similar to our prior findings, in which we compared tumors with and without a successful genetic analysis. For instance, an unknown chromosome 3 status may occur when only a small piece of tumor material was available for chromosome analysis or when the eye was removed after prior irradiation, as we reported a few years ago.[56]

In conclusion, chromosome aberrations had more impact when the patient had a light iris than when the patient had a brown iris. Gaining knowledge on the pigmentation pathways in the pathogenesis of 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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Abbreviations and Acronyms:

D3 = disomy of chromosome 3; **LUMC** = Leiden University Medical Center; **M3** = monosomy of chromosome 3; **SNP** = single nucleotide polymorphism; **UM** = uveal melanoma; **WEH** = Wills Eye Hospital.

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