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Genetic prognostication in uveal melanoma

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Chapter 4

The prognostic value of AJCC Staging in Uveal Melanoma is enhanced by adding chromosome 3 and 8q status

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ABSTRACT

Purpose: The American Joint Committee on Cancer (AJCC) staging system has been validated for use as a prognostic parameter in uveal melanoma (UM). We studied whether adding information regarding chromosome 3 and 8q status further enhances the prognostic value of this staging system.

Methods: We retrospectively studied a cohort of 522 patients who had been treated for UM in two different centers between 1999 and 2015. The mean follow-up time was 47.7 months. Cumulative incidence curves were generated and regression analyses were performed for different combinations of AJCC staging and chromosome status. Death due to UM metastases was the primary endpoint.

Results: In AJCC stage I cases, only patients with monosomy 3 as well as chromosome 8q gain died due to UM metastases ($P < 0.001$). Among patients with stage II and III tumors, those with monosomy 3 plus gain of chromosome 8q had the worst prognosis, whereas the clinical outcome of those with only one of these aberrations was intermediate ($P < 0.001$). Patients without monosomy 3 and 8q gain showed favorable prognosis, independent of their tumor's AJCC stage. In cases with monosomy 3, 8q gain, or both, adding AJCC stage improved the predictive value. Multivariable regression analyses demonstrated that AJCC staging and chromosome 3 and 8q status contain independent information about survival status.

Conclusions: Combining information on AJCC staging and chromosome 3 and 8q status allows a more accurate prognostication in UM. We conclude that the prognostic value of the AJCC staging system can be improved by adding information regarding chromosome 3 and 8q status.

INTRODUCTION

Prognostication in uveal melanoma (UM) is of importance for patient counseling and stratification of patients in clinical trials. A variety of patient and tumor characteristics can be used for prognostication in UM. Parameters such as tumor size, tumor location, and extraocular growth have been identified as prognostic factors¹⁻⁴ and this has resulted in the Tumor-Node-Metastasis (TNM) classification. The American Joint Committee on Cancer (AJCC) Cancer Staging Manual uses the TNM classification to describe the different stages of various types of cancer. The first edition, in which stages were defined for prognostication in UM, was published in 1983.⁵ The current seventh edition of the AJCC staging uses tumor size, ciliary body involvement, and extraocular growth as its parameters for prognostication in UM.^{6,7} Although the AJCC staging system for UM is an internationally recognized and validated method to estimate patient survival, it is currently based only on the anatomic extent of the tumor and does not take genetic data into account.⁸⁻¹⁰

Concurrent to the development and refinement of the classical AJCC staging system, translational research has led to more insight into the genetics of UM. Chromosome abnormalities, such as monosomy of chromosome 3,¹¹⁻¹³ amplification of chromosome 8q,¹⁴⁻¹⁷ loss of chromosome 1p,¹⁸ and gain of chromosome 6p,¹⁹ have been identified as prognostic parameters. Especially monosomy 3 (M3) and polysomy of 8q are strongly correlated with the development of metastatic disease, whereas polysomy of chromosome 6p in the absence of changes in chromosomes 3 and 8 is indicative of a favorable prognosis. Besides chromosome abnormalities, gene-expression profiling, which divides UMs into class 1 (associated with low metastatic risk) and class 2 (associated with high metastatic risk) tumors,^{20,21} can be used for prognostication. Recently, the prognostic importance of mutations in specific genes, such as *BAP1*, *EIF1AX*, and *SF3B1*, has come to light.²²⁻²⁵

Both types of prognostic tumor features, anatomic and genomic, are currently used for risk stratification of UM patients in trials. Damato and associates²⁶ were the first to develop a multifactorial algorithm to generate individualized prognostic curves for UM patients. This algorithm takes into account patient age and sex, the TNM size category of the tumor, as well as histological features and chromosome 3 loss.^{26,27} Other authors have since also addressed the question as to whether the combined use of the TNM/AJCC staging system and genomic characteristics of the tumor will allow for better risk stratification.^{28,29} As

proposed earlier,^{8,26,27} integrating the TNM/AJCC staging system with genetics may enhance prognostication in UM.

This is supported by the findings of a recent study by Bagger et al.³⁰ in a Danish cohort of 153 patients, which indicated that AJCC stage III and aberrations in chromosome 3 and 8 are independent prognostic factors. However, the relatively low number of cases limited this study, as several risk groups did not contain sufficient cases to perform reliable statistical analyses.

By merging the data from the Leiden University Medical Center (LUMC), The Netherlands, on 275 enucleated UMs and the prior as well as more recent data of the Copenhagen University Hospital Rigshospitalet, Denmark, we were able to investigate the effect of combining information regarding the AJCC staging and chromosome 3 and 8q status on prognostication in a joint cohort of 522 patients. We hypothesize that prognostication by the AJCC staging system can be enhanced by adding information on the chromosome 3 and 8q status of the tumor.

METHODS

Patients

Between January 8, 1999, and December 19, 2013, a total of 366 patients underwent a primary enucleation for ciliary body and/or choroidal melanoma at the LUMC, which has been acknowledged as top referral center for ocular melanoma by the Dutch Federation of University Medical Centers. The 275 cases in which the chromosome 3 status was determined are included in this study. The Danish cohort consists of 247 patients treated from January 1, 2009, through July 21, 2015, at the national referral center at Copenhagen University Hospital, which is the referral hospital for patients in East Denmark. In Denmark, there is one national referral center at two different locations: one for East Denmark and one for West Denmark.

Primary enucleation was performed in 89 patients, whereas 156 patients underwent brachytherapy, one patient underwent tumor resection, and one patient refused treatment.

Genetic information on both chromosome 3 and 8q was available in 225 Danish cases and in 245 Dutch cases. This yielded a total number of 470 cases that were available for survival analyses in this study (Fig. 1).

Survival data of Dutch patients was obtained from the Integral Cancer Center West, a regional office of the Netherlands Comprehensive Cancer Organisation (<https://iknl.nl/over-iknl/about-iknl>), which registers information about every

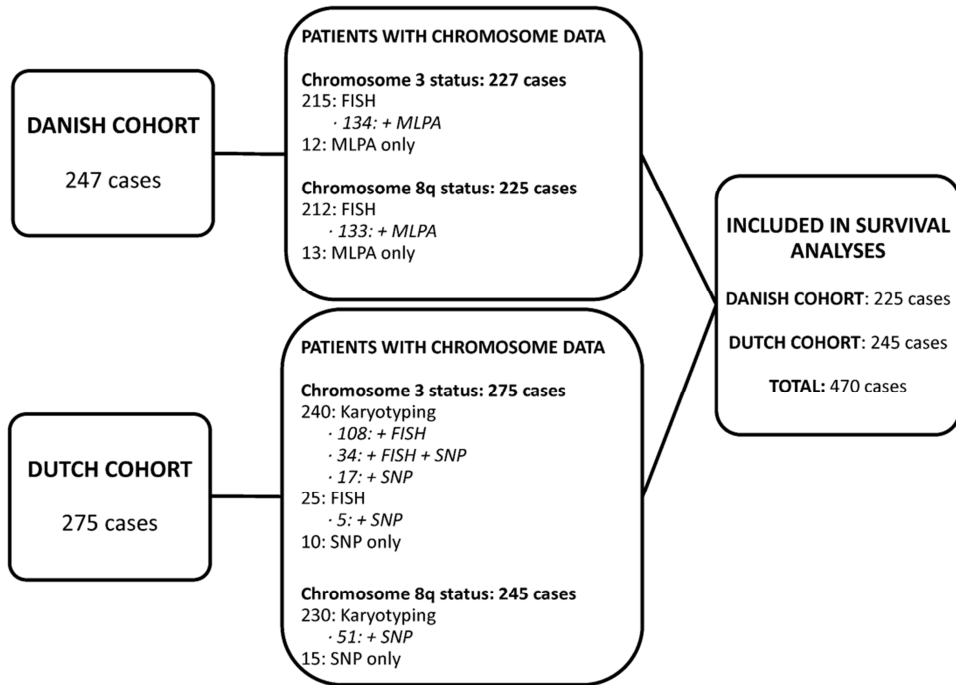


Figure 1. Number of cases with available chromosome data.

cancer patient. The occurrence of metastases and the survival status of patients are checked on a yearly basis, based on information provided by the general practitioner and/or the hospital. In most cases, the diagnosis of metastases is based on the clinical evaluation of the patient by the general practitioner. Because there is no effective treatment for metastatic UM, follow-up in The Netherlands is neither strictly regulated nor intensive and patients are referred back to their general practitioner. Imaging and histologic examination to confirm the diagnosis of metastases were done in only a minority of patients in whom the clinical diagnosis was equivocal or in case patients participated in a clinical trial. Follow-up was last updated in February 2016.

In the Danish cohort, all patients were offered a physical examination, liver function tests, radiography of the thorax, and liver ultrasonography at 3, 6, 12, 18, 24, 30, 36, 48, 60, 84, and 120 months posttreatment. Magnetic resonance imaging (MRI) or a computed tomography (CT) scan was performed when metastatic spread was suspected. When the MRI or CT scan was positive, an additional positron emission tomography (PET)-CT scan was performed. Metastases limited to the liver were biopsied for histopathologic and

immunohistochemical examination. Otherwise, a biopsy of the most easily accessible site was taken. The referral center was immediately informed when a patient died. The survival status was last updated in March 2016. No patients were lost to follow-up in either of the cohorts.

The Regional Research Ethics Committees in both centers waived the need for approval of this retrospective cohort study. This study adhered to the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964, ethical principles for medical research involving human subjects).

Histologic Examination

In both centers, enucleated eyes and biopsies were fixed in 4% neutral-buffered formalin for 48 hours and embedded in paraffin. Hematoxylin-eosin–stained sections of 4- μ m thickness were assessed by an ocular pathologist to confirm the diagnosis and to determine histologic tumor characteristics.

American Joint Committee on Cancer Staging

The location of the tumor in the eye, the largest basal diameter (LBD, in millimeters), thickness (in millimeters), and the presence of extraocular extension (≤ 5 or > 5 mm) were evaluated in histological specimens for all patients in the Dutch cohort and for enucleated cases of the Danish cohort. In Denmark, the AJCC staging was determined clinically (diaphanoscopy, ultrasonography, B-scan, and MRI) and additional histologic evaluation was done in the 89 enucleated tumors. Transvitreal retinochoroidal biopsies were obtained in 235 patients.³⁰

Cytogenetic Analysis

Between 1999 and 2013, a total of 291 UMs enucleated at the LUMC were sent in for karyotyping with or without fluorescence in situ hybridization (FISH).³¹ In 62 of these cases and four other tumors, single nucleotide polymorphism (SNP) array was performed as well.

The regulations of the International System for Human Cytogenetic Nomenclature, 1995, were used for describing the karyotype. Fluorescence in situ hybridization was performed with DNA probes specific for the centromere of chromosome 3 (probe: α -sat3; Cytocell, Cambridge, UK) and for region 3p24.3-p25 (probe: RP11–322M13; Cytocell).

A tumor was designated as having M3 or gain of chromosome 8q on the karyogram when this abnormality was observed in at least two cells. Alternatively,

for M3, the presence of this aberration in only one cell was sufficient provided it was accompanied by other chromosome abnormalities characteristic of UM. When the chromosome aberration resulting in the gain of chromosome 8q was an isochromosome of 8q, the presence of this aberration in only one cell was sufficient.

Two types of SNP microarray chips were used: the Affymetrix 250K_NSP-chip, with approximately 250,000 probes across the genome, and the Affymetrix Cytoscan HD chip, with approximately 750,000. The “Genotyping Console (GTC)” was used to determine the copy numbers 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 with “Chromosome Analysis Suite (ChAS).” To adjust for partial gains or deletions, different loci per chromosome were evaluated. Approximately 200 probes per gene locus were averaged to determine copy numbers.

In cases of disagreement among karyotyping, FISH, and SNP, tumors were designated as having M3 or chromosome 8q gain when either of the tests showed the abnormality.

In the Danish cohort, the chromosome status was evaluated in 235 tumors by FISH and multiplex ligation-dependent probe amplification (MLPA).

Fluorescence in situ hybridization was performed using centromeric probes for chromosomes 3 (CEP3 D3Z1) and 8 (CEP8) (both probes from Abbott Molecular, Inc., Des Plaines, IL, USA; www.abbottmolecular.com [in the public domain]). A minimum of 100 cells from each specimen were evaluated when possible.

Cytogenetic abnormalities were reported when at least 10% of the analyzed cells showed the abnormality.

Multiplex ligation-dependent probe amplification analysis (SALSA MLPA P027 Uveal melanoma; MRC-Holland, Amsterdam, The Netherlands) was performed on tumor tissue from all patients treated between 2012 and 2015, and retrospectively in patients treated between 2009 and 2011, of whom tumor tissue was available from snap-frozen biopsies.

In cases of disagreement between FISH and MLPA, a tumor was categorized as an M3 tumor or one having chromosome 8q gain when one of the tests showed the chromosome abnormality.

Statistical Analysis

For data analysis, the statistical software package SPSS v. 20.0.0 (IBM SPSS

Statistics for Windows; IBM Corp., Armonk, NY, USA) was used. Characteristics of the study population were described by percentages, means and SDs. Pearson's χ^2 tests, Student's *t*-tests, and Linear-by-Linear Association tests were performed to evaluate statistical differences between the Danish and the Dutch cohorts. The association between AJCC staging and chromosome 3 and 8q status was analyzed using the Linear-by-Linear Association test.

Cumulative incidence curves were computed, which accounted for death by other causes than UM metastases as a competing risk. Gray's K-sample test was performed to evaluate statistical significance.³² The Bonferroni correction was applied for pairwise comparisons; *R* version 3.1.3 (R Development Core Team, Vienna, Austria) was used for computing the cumulative incidence curves using package *cmprsk* version 2.2.³³ Cumulative incidence rates of death due to UM metastases (with 95% confidence intervals [CIs]) for 5 years of follow-up were calculated.

Hazard ratios (HRs) of death due to UM metastases by AJCC stage and chromosome status were estimated in Cox regression models of the events of melanoma-related death by censoring for end of follow-up or death due to other causes. Time since initial treatment of UM was used as time scale and both unadjusted and adjusted analyses were conducted. In the adjusted analysis, we furthermore accounted for sex and age at treatment. Effect estimates are reported as HRs with 95% CIs. To evaluate the effect of competing risks in the regression model, we also performed a competing risks regression analysis using the Fine and Gray model,³⁴ which extends the Cox model to account for several causes of death. The Fine and Gray model³⁴ also was used to generate predictions of incidence of UM-related death at 5 years of follow-up for subgroups of patients based on AJCC stage and chromosome status. The Fine and Gray model³⁴ was applied to evaluate possible changes in interpretation due to the competing risk situation and the Cox model was included for ease of interpretation. All statistical tests were two-sided and based on the likelihood ratio test. A significance level of 5% was applied. The statistical software *R* (base package survival)³³ was used to apply the Fine and Gray model.³⁴

RESULTS

Population Characteristics

The combined cohort from the two oncology centers resulted in a group of 522 patients, of whom 49% were females. The mean age at treatment of the primary

tumor was 61.9 years. The Danish and Dutch cohorts did not differ significantly regarding sex and age. Concerning AJCC staging, 17% of the tumors were stage I, more than half of the tumors (59%) were classified as stage II, whereas 23% were stage III, and 1% stage IV. The Dutch cohort contained more tumors of higher AJCC stages ($P = 0.005$) (Table 1).

Table 1. Characteristics of the study population. The characteristics of the Danish and the Dutch cohorts are depicted separately. Possible statistical differences between the two cohorts were evaluated. Significant P values are in bold. Percentages are rounded and may not total 100.

Characteristic	Danish Cohort <i>n</i> = 247	Dutch Cohort <i>n</i> = 275	<i>P</i> Value	Combined Cohort <i>n</i> = 522
Females, <i>n</i> (%)	132 (53)	124 (45)	0.06*	256 (49)
Mean age at primary treatment, \bar{y} (\pm SD)	61.9 (\pm 14.1)	61.9 (\pm 13.7)	0.99†	61.9 (\pm 13.9)
Mean tumor diameter, mm (\pm SD)	12.6 (\pm 4.2)	12.1 (\pm 3.5)	0.18†	12.3 (\pm 3.8)
Mean tumor thickness, mm (\pm SD)	5.6 (\pm 3.2)	7.0 (\pm 3.0)	<0.001†	6.4 (\pm 3.2)
Extraocular growth, <i>n</i> (%)	8 (3)	27 (10)	0.003*	35 (7)
Ciliary body involvement, <i>n</i> (%)	56 (23)	113 (41)	<0.001*	169 (32)
AJCC stages, <i>n</i> (%)				
I	60 (24)	31 (11)		91 (17)
II	135 (55)	175 (64)		310 (59)
III	49 (20)	69 (25)		118 (23)
IV	3 (1)	0 (0)	0.005‡	3 (1)
Monosomy 3, <i>n</i> (%)	120 (53) <i>(known in 227 cases)</i>	146 (53) <i>(known in all cases)</i>	0.96*	266 (53) <i>(known in 502 cases)</i>
Chromosome 8q gain, <i>n</i> (%)	108 (48) <i>(known in 225 cases)</i>	115 (47) <i>(known in 245 cases)</i>	0.82*	223 (47) <i>(known in 470 cases)</i>
Metastases, <i>n</i> (%)	48 (19)	101 (37)	<0.001*	149 (29)
Survival, <i>n</i> (%)				
Death due to UM metastases	43 (17)	89 (32)		132 (25)
Death due to other reasons	20 (8)	41 (15)		61 (12)
Alive at last follow-up date	184 (74)	145 (53)	<0.001*	329 (63)
Mean follow-up length in months (\pm SD)	39.0 (\pm 23.0)	55.5 (\pm 41.1)	<0.001†	47.7 (\pm 34.8)

Symbols: * Pearson's χ^2 , † Student's *t*-test, ‡ Linear-by-Linear Association

The chromosome 3 status was successfully determined in 227 cases of the Danish cohort, whereas the chromosome 8q status was known in 225 tumors. Regarding the Dutch cohort, the chromosome 3 status was known in all cases and the chromosome 8q status in 245 tumors. The cohorts did not differ significantly regarding the percentage of tumors harboring M3 and gain of chromosome 8q ($P = 0.96$ and $P = 0.82$, respectively).

The mean follow-up time was 55.5 months (range, 1–193) for the Dutch cohort and 39 months (range, 2–86) for the Danish cohort, and the combined follow-up time was 47.7 months (Table 1). At last follow-up, 149 (29%) patients had developed metastases; 3 patients already had metastases at the time of diagnosis of the primary tumor. During the follow-up period, 132 patients (25%) died due to UM metastases. The metastasis rate was higher in the Dutch cohort ($P < 0.001$) and a higher percentage of the Dutch patients died due to UM metastases ($P < 0.001$).

Cumulative Incidence Analysis: AJCC Staging and Chromosome 3 and 8q Status

Cumulative incidence curves were generated for the AJCC stages and the chromosome profiles (Fig. 2). Subcategories of the AJCC stages were not used. AJCC stage IV was not included in the survival analyses, because the number of patients having this stage ($n = 3$) was insufficient for a reliable analysis. Patients with only M3 or only chromosome 8q gain were combined in a single group because they did not differ significantly in survival (Supplementary Fig. S1), and by combining these cases we were able to create a single group with larger patient numbers, increasing the power of the analysis.

The cumulative incidence analysis for the AJCC staging showed a clearly distinct risk of death due to UM metastases for each of the different AJCC stages ($P < 0.001$) (Fig. 2A). The incidence of UM death was highest in patients with AJCC stage III tumors ($P < 0.001$, pairwise comparison versus AJCC stage II with Bonferroni correction).

The various chromosome profiles were associated with a different incidence of metastatic death ($P < 0.001$) (Fig. 2B). The incidence of death from UM metastases was highest in patients with M3 as well as chromosome 8q gain ($P < 0.001$, pairwise comparison versus “M3 OR 8q gain” group with Bonferroni correction), whereas patients with either M3 or chromosome 8 gain showed an intermediate prognosis ($P = 0.01$, pairwise comparison versus “No M3 AND No 8q gain” group with Bonferroni correction).

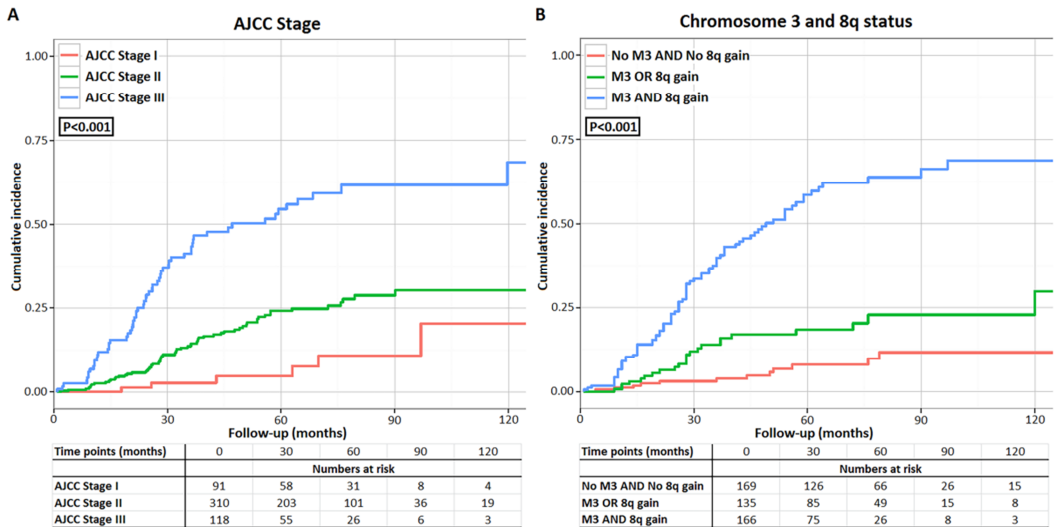


Figure 2. Uveal melanoma–related death in relation to different AJCC stages and chromosome status. Cumulative incidence curves are shown of different AJCC stages and chromosome 3 and 8q profiles in the total cohort. (A) AJCC stage; (B) Chromosome 3 and 8q status.

Table 2. The distribution of patients according to AJCC stage and chromosome 3 and 8q status. Only cases of which the chromosome 3 as well as the chromosome 8q status was known are included. The Linear-by-Linear Associate test was applied.

AJCC Stage	Chromosome 3 and 8q Status				Total	P Value
	No M3 and No 8q Gain	Only 8q Gain	Only M3	M3 and 8q Gain		
I	42	3	17	13	75	<0.001
II	111	42	44	86	283	
III	16	10	17	66	109	
IV	0	2	0	1	3	
Total	169	57	78	166	470	

Cumulative Incidence Analysis: Adding Chromosome 3 and 8q Status to AJCC Staging

Higher AJCC stages were associated with prognostically poor chromosome profiles ($P < 0.001$) (Table 2). To evaluate the effect of chromosomes 3 and 8q on survival when the cohort is stratified by AJCC staging, we conducted separate cumulative incidence analyses in tumors of the three AJCC stages (Fig. 3).

Of the patients with stage I tumors, only patients with tumors having M3 as well as 8q gain died due to disseminated disease ($P < 0.001$) (Fig. 3A). The 5-year

cumulative incidence of death due to UM metastases was 25% (95% CI 3%–59%) (Table 3).

Table 3. The observed 5-year cumulative incidence rates of death due to UM metastases according to AJCC stage and chromosome 3 and 8q status. The 95% CIs are indicated between parentheses.

AJCC Stage	Chromosome 3 and 8q Status		
	No M3 and No 8q Gain, %	M3 or 8q Gain, %	M3 and 8q Gain, %
I	0	0	25 (3–59)
II	11 (5–19)	17 (9–27)	50 (37–62)
III	9 (0–35)	32 (14–52)	73 (58–83)

Stage II tumors showed a difference in incidence of UM-related death among the three chromosome status groups ($P < 0.001$) (Fig. 3B), with the highest incidence of death due to UM metastases occurring in patients with M3 as well as 8q gain (5-year incidence: 50%, 95% CI 37%–62%) ($P = 0.002$, pairwise comparison versus “M3 OR 8q gain” group with Bonferroni correction). The prognosis in cases with either a normal chromosome status (5-year incidence: 11%, 95% CI 5%–19%) or with M3 or chromosome 8q gain only (5-year incidence: 17%, 95% CI 9%–27%) was intermediate and comparable ($P = 0.54$ with Bonferroni correction). A similar pattern was observed in stage III tumors ($P < 0.001$) (Fig. 3C): patients with a tumor having M3 as well as chromosome 8q gain had the most unfavorable outcome (5-year incidence: 73%, 95% CI 58%–83%) ($P < 0.001$, pairwise comparison versus “M3 OR 8q gain” group with Bonferroni correction). Additionally, the prognostic effect of adding only information regarding the chromosome 3 status or the chromosome 3 status combined with the chromosome 8q status was analyzed (Figs. 3D–F). The red curves in each of the three graphs depict the incidence of UM-related death in all cases of the respective AJCC stage. In case only information on chromosome 3 had been available, stratification would have been possible (olive green [M3] and blue [No M3] graphs). The additional effect of also including information on the status of chromosome 8q is shown by the green (M3 AND 8q gain) and purple (No M3 AND No 8q gain) graphs.

Cumulative Incidence Analysis: Adding AJCC Staging to Chromosome 3 and 8q Status

In addition, we investigated whether adding information on the AJCC staging would increase prognostic accuracy when the chromosome status is known. Therefore, we performed cumulative incidence analyses in the three groups with different chromosome profiles, using the AJCC stage as the factor of interest (Supplementary Fig. S2).

Considering only patients having a tumor without M3 and without chromosome 8q gain, none with a stage I tumor died due to metastases, whereas stage II and stage III tumors showed a comparable incidence of UM deaths ($P = 0.13$) (Supplementary Fig. S2A) (5-year incidence: see Table 3).

Regarding tumors with either M3 or chromosome 8q gain, the incidence of UM death in patients with a stage II or a stage III tumor was largely comparable, whereas none of the stage I cases died due to UM metastases ($P = 0.03$) (Supplementary Fig. S2B). In tumors with M3 as well as chromosome 8q gain, the incidence of death due to UM metastases was clearly higher in patients with stage III tumors when compared with those with stage I or stage II tumors ($P < 0.001$) (Supplementary Fig. S2C).

Regression Analyses

We performed a competing risks regression analysis based on the Fine and Gray model³⁴ for the parameters of sex, age at treatment, AJCC stage, and chromosome status (Table 4). Sex and age were not significantly associated with UM-related death. American Joint Committee on Cancer stage III (regression coefficient: 8.05) and the combination of M3 and chromosome 8q gain (regression coefficient: 6.83) were the characteristics with the largest regression coefficients (both $P < 0.001$). As expected, age at treatment was associated with the risk of death due to other causes.

Additionally, univariate and multivariable Cox regression analyses were performed. All factors, except sex and age, were significantly associated with melanoma-related death in the multivariable model (Supplementary Table S1). American Joint Committee on Cancer stage III tumors (HR 8.8, 95% CI 2.73–28.39, $P < 0.001$) and tumors with M3 as well as 8q gain (HR 7.95, 95% CI 4.24–14.89, $P < 0.001$) showed the largest HRs, which is in concordance with the results of the Fine and Gray model.³⁴

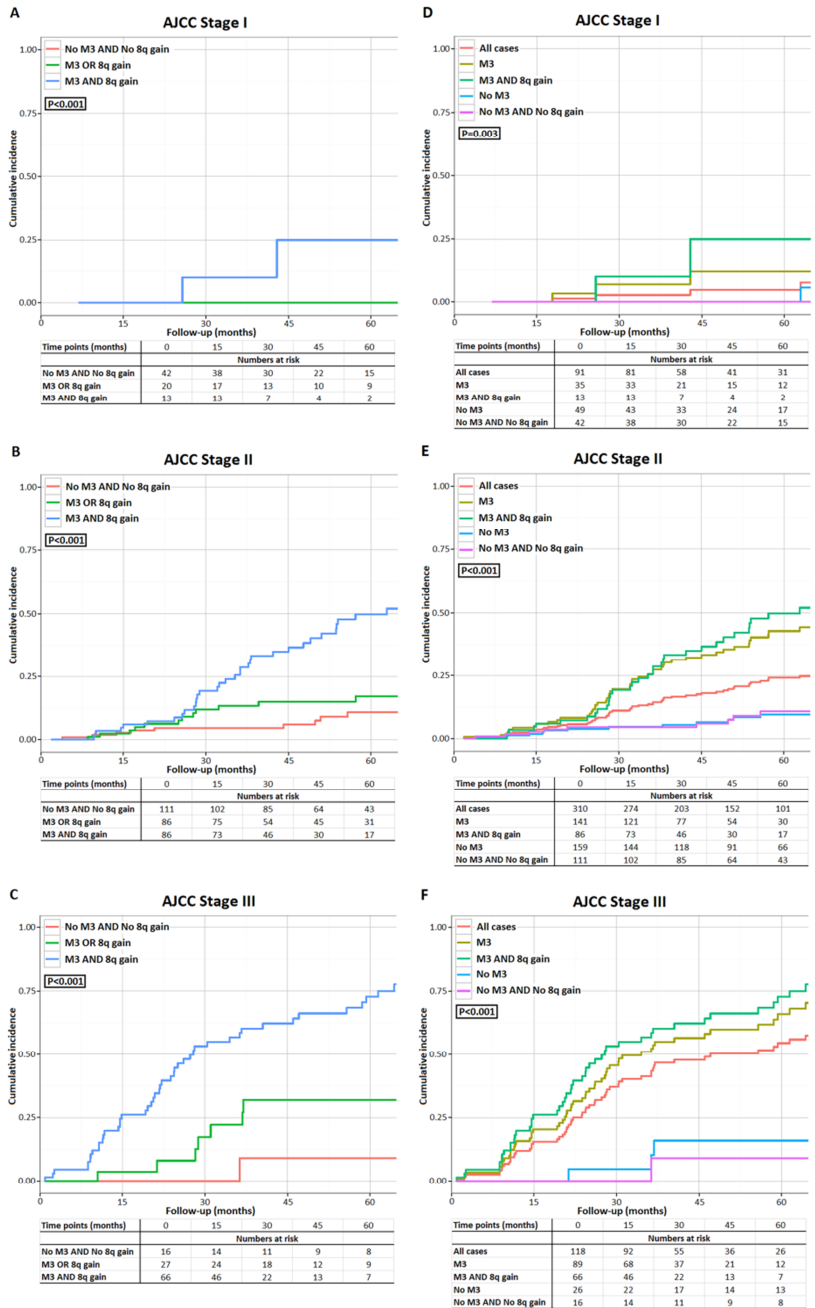


Figure 3. Incidence of UM-related death for the chromosome profile groups, analyzed in the different AJCC stages. *First column:* the effect of chromosome 3 and 8q status on the incidence of UM-related death in different AJCC stages ([A] AJCC stage I; [B] AJCC stage II; [C] AJCC stage III). *Second column:* the effect of adding only the chromosome 3 status or the chromosome 3 status combined with the chromosome 8q status to tumors of different AJCC stages ([D] AJCC stage I; [E] AJCC stage II; [F] AJCC stage III).

Table 4. Competing risks regression analysis based on the Fine and Gray model.

Significant p-values are in bold.

Characteristic	UM Death			Other Death		
	Regression Coefficient	95% CI	P Value	Regression Coefficient	95% CI	P Value
Female gender	1.06	0.72 – 1.55	0.78	0.74	0.42 – 1.3	0.3
Age at treatment	1.01	0.99 – 1.02	0.25	1.05	1.02 – 1.08	<0.001
AJCC stage I (reference category)	-			-		
AJCC stage II	3.79	1.27 – 11.32	0.02	1.16	0.52 – 2.6	0.72
AJCC stage III	8.05	2.66 – 24.43	<0.001	0.57	0.21 – 1.53	0.26
No M3 AND no 8q gain (reference category)	-			-		
M3 OR 8q gain	2.1	1.03 – 4.27	0.04	1.36	0.68 – 2.73	0.39
M3 AND 8q gain	6.83	3.64 – 12.81	<0.001	1.47	0.72 – 2.97	0.29

Multivariable Estimates of Incidence of UM-Related Death

The Fine and Gray model³⁴ was used to generate estimates of incidence of death due to UM metastases at 5 years of follow-up (Table 5). These largely correspond with the observed estimates of cumulative incidence of UM-related death at 5 years of follow-up in our cohort (Table 3).

Table 5. The estimated 5-year incidence rates of death due to UM metastases according to AJCC stage and chromosome 3 and 8q status. The Fine and Gray model³⁴ was applied. The 95% CIs are indicated between parentheses.

AJCC Stage	Chromosome 3 and 8q Status		
	No M3 and No 8q Gain, %	M3 or 8q Gain, %	M3 and 8q Gain, %
I	0	0	30 (0–54)
II	11 (4–17)	19 (9–27)	45 (32–55)
III	7 (0–20)	34 (12–51)	77 (64–85)

DISCUSSION

This international retrospective cohort study in 522 patients with primary UM adds further evidence to our previously published results³⁰ and shows that combining two internationally recognized prognostication methods, AJCC

staging^{6,9,10} and status of chromosomes 3 and 8q,^{11–17} enhances the stratification between low-risk and high-risk patients.

The combination of both the anatomic extent and genetic status has previously been shown by Damato et al.^{26,27} to be a valid method for prognostication. The parameters in their prognostic model, Liverpool Uveal Melanoma Prognosticator Online (LUMPO), include largest tumor diameter, largest tumor height, which correspond to the AJCC tumor size, extraocular extension, and anterior tumor margin, which often will correspond to ciliary body involvement. Additional parameters in Damato's model are tumor cell type, presence of extravascular closed-loop matrices, and mitotic count.²⁶

The current study takes advantage of the established TNM cancer staging system, which adheres to general anatomical staging principles only, and combines these data with additional genetic information of the tumor cells, obtained by karyotyping, FISH, SNP, and MLPA.

In our current study, the cumulative incidence of UM-related death increased in higher AJCC stages, and patients with a tumor having M3 as well as chromosome 8q gain had a poorer prognosis than those with either one of the aberrations or no aberrations at all. This is in accordance with findings of an earlier study.³⁵ Although there was an association between higher AJCC stage and the frequency of chromosome aberrations (Table 2), we found that combining the AJCC staging and the chromosome status provides additional information regarding UM-related death (Fig. 3; Supplementary Fig. S2).

Interestingly, in patients with an AJCC stage I tumor, the only patients dying of metastatic UM were those with the worst genetic profile: a combination of M3 and chromosome 8q gain. This would suggest that in case of a tumor with a limited anatomic extent, the combination of M3 and chromosome 8q gain is required for these smaller tumors to metastasize. Only one of these chromosome aberrations does not seem to be sufficient to cause metastatic spread of these tumors, at least not during the follow-up period of our study. However, for AJCC stage II as well as stage III tumors, patients with a normal chromosome status or with either M3 or chromosome 8q gain did die due to UM metastases. Apparently, tumors of higher AJCC stages already possess a particular degree of malignancy that appears to affect survival independently of chromosome status. Nevertheless, even in these larger tumors, patients having M3 as well as chromosome 8q gain had the worst prognosis. The fact that a combination of these chromosome aberrations results in a worse survival than either of these aberrations separately

has been described previously³⁵; however, to our knowledge, there are no studies demonstrating this specifically in the largest tumors (AJCC stage III).

We also analyzed whether AJCC staging has an additive effect on the predictions made on the basis of the tumor's chromosome status. In tumors with a normal chromosome 3 and 8q status, AJCC staging did not have a significant effect. These tumors may have such a prognostically favorable genetic profile that the anatomic extent has only a minor effect on survival. In tumors with either M3 or chromosome 8q gain, the difference in cumulative incidence of UM-related death between stage II/stage III tumors and stage I cases was obvious, whereas little difference was observed between stage II and stage III tumors. In the group with M3 as well as chromosome 8q gain, the effect of AJCC staging on the incidence of UM-related death was even more apparent. The incidence was clearly highest in patients with an AJCC stage III tumor. Although metastatic death occurs in tumors of all AJCC stages in this group of tumors, the AJCC stage III tumors are located at the far end of the spectrum regarding prognosis. Presumably, these larger tumors have had a longer time to develop (lead-time bias) and may have accumulated additional chromosome aberrations resulting in increased malignant behavior. A recent study by Corrêa and Augsburger²⁸ reported that gene-expression profiling and LBD are independent prognostic factors for death due to UM metastases. To evaluate whether combining information on LBD (cutoff 12 mm, the median value in the study by Corrêa and Augsburger²⁸) and chromosome 3 and 8q status provides similar risk stratification as combining AJCC staging and chromosome 3 and 8q status, we performed additional analyses (Supplementary Fig. S3). We found that combining AJCC staging and chromosome 3 and 8q status results in better risk stratification than the combination of LBD (cutoff 12 mm) and chromosome status. When only LBD with the cutoff of 12 mm is considered, the smallest tumors (AJCC stage I) without M3 and without chromosome 8q gain or with only one of these aberrations, and which do not lead to death due to UM metastases (at least not during our follow-up period) are not identified anymore as a separate group. All cases with LBD \leq 12 mm die due to UM metastases, regardless of chromosome profile (Supplementary Fig. S3A). Moreover, the additional prognostic effect of LBD is less obvious and not statistically significant in the group with M3 as well as 8q gain. Although there is a distinct difference in the incidence of UM-related death in this group between various AJCC stages, especially between stage II and III (Supplementary Fig. S2C), this difference is less evident when LBD with a cutoff of 12 mm is considered (Supplementary Fig. S3E).

To evaluate the mutual contributions of predictors of melanoma-related death and to take into account the effect of competing risks, a competing risks regression analysis based on the Fine and Gray model³⁴ was performed. American Joint Commission on Cancer stage III and the combination of M3 and chromosome 8q gain showed the highest regression coefficients in this multivariable competing risks model. This indicates that these risk factors contain independent information about survival status. In accordance with the Fine and Gray model,³⁴ HRs for the risk factors AJCC staging and chromosome 3 and 8q status remained statistically significant in the multivariable Cox regression model.

The Fine and Gray model³⁴ also was used to calculate the estimated incidence of UM-related death at 5 years of follow-up. This was largely in agreement with the observed values in our cohort, supporting the validity of the regression coefficients of this multivariable model.

Our data show that adding chromosome status improves prognostication by AJCC staging and that AJCC staging is of additional prognostic value when aberrations in chromosome 3 and 8q status are observed. This has several implications for prognostication in UM.

First, this knowledge may be used to further improve the AJCC staging system. The AJCC staging system has been evolving and has become more sophisticated over the years. Although the seventh edition and the newly published eighth edition of the AJCC staging system do not include genomic information into their classification system, the authors of these UM chapters mention that additional nonanatomical parameters are important in UM, and that these data should be collected as either biomarkers or data points. Including genetic parameters may be especially valuable for the staging of UM, because certain genetic aberrations such as M3 and chromosome 8q gain are strongly correlated with poor prognosis. The results of our study indicate that including information on the chromosome 3 and 8q status into the AJCC staging system might be worthwhile.

Second, it shows that it is useful to add information on the AJCC stage when the status of chromosome 3 and 8q is known. Besides chromosome status, gene-expression profiling has become a valuable tool for prognostication in UM.^{36,37} As it closely corresponds to chromosome 3 status, it is likely that addition of the AJCC stage could improve the prognostic value of gene-expression profiling as well. The idea that AJCC staging could refine prognostication by gene-expression analysis has already been proposed by Kivelä and Kujala in 2013.⁸

The strength of our study is that we have analyzed a large international cohort of patients with accurate follow-up. Medical charts and pathology reports were reviewed to check the cause of death, in addition to the information reported by the cancer registries.

Tumors that were irradiated comprised a major part of the Danish cohort, whereas the Dutch cohort consisted of only enucleated tumors. Because only small tumors are eligible for radiation treatment, the Danish tumors were thinner and were more often categorized in lower AJCC stages than the Dutch tumors; however, combining these two cohorts has resulted in a heterogeneous joint cohort. This makes the results of our study applicable to irradiated as well as enucleated UM. Furthermore, in both centers, the chromosome status was determined by using two techniques, which yields results that are more reliable. A problem with karyotyping combined with FISH on cultured cells is that of the 291 cases that were sent for analysis, 26 (9%) UMs were not evaluable and no reliable result was obtained. Genetic analysis in the LUMC is now performed using SNP arrays, but this technique was performed in only 66 cases in this cohort. The main limitation of our study is that we included a considerable number of recently diagnosed and treated patients, who obviously have a short follow-up. This limited our ability to identify patients dying due to UM metastases late after diagnosis. This was particularly the case for the Danish cohort, which showed a lower incidence of disease-specific death due to lower AJCC stage tumors and shorter follow-up.

Although the Dutch cohort starts in 1999, most included patients (189 of 275, 68%) were treated in the period between 2007 and 2013, which explains why the mean Dutch follow-up time was not much longer than the follow-up time of the Danish cohort. Additionally, although both centers used two techniques to increase reliability of the results, the fact that the two centers used different genetic tests with diverse sensitivities may have caused a variation in the detection rate of the chromosome aberrations of interest. The fact that the frequency of M3 and chromosome 8q gain between the two centers is similar, despite more metastases and more advanced-stage tumors in the Dutch cohort, may indicate that some aberrations in chromosomes 3 and 8q could have been missed in the Dutch tumors. However, no statistical difference in survival was found between Danish and Dutch tumors without M3 and neither in tumors without M3 and without 8q gain (Supplementary Fig. S4).

In summary, the present study shows that AJCC staging and chromosome 3 and 8q status yield additional information regarding prognosis in UM. This provides an opportunity to improve the prognostication of patients with UM. Future AJCC staging systems for UM would be enhanced by the inclusion of data concerning chromosomal copy number variations and gene mutations.

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Competing Interests Statement

All authors declare that no conflicts of interest exist.

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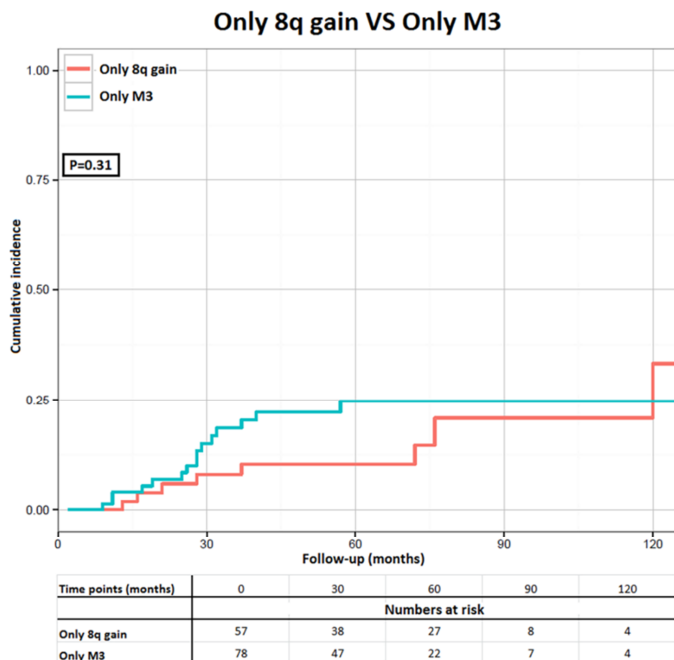
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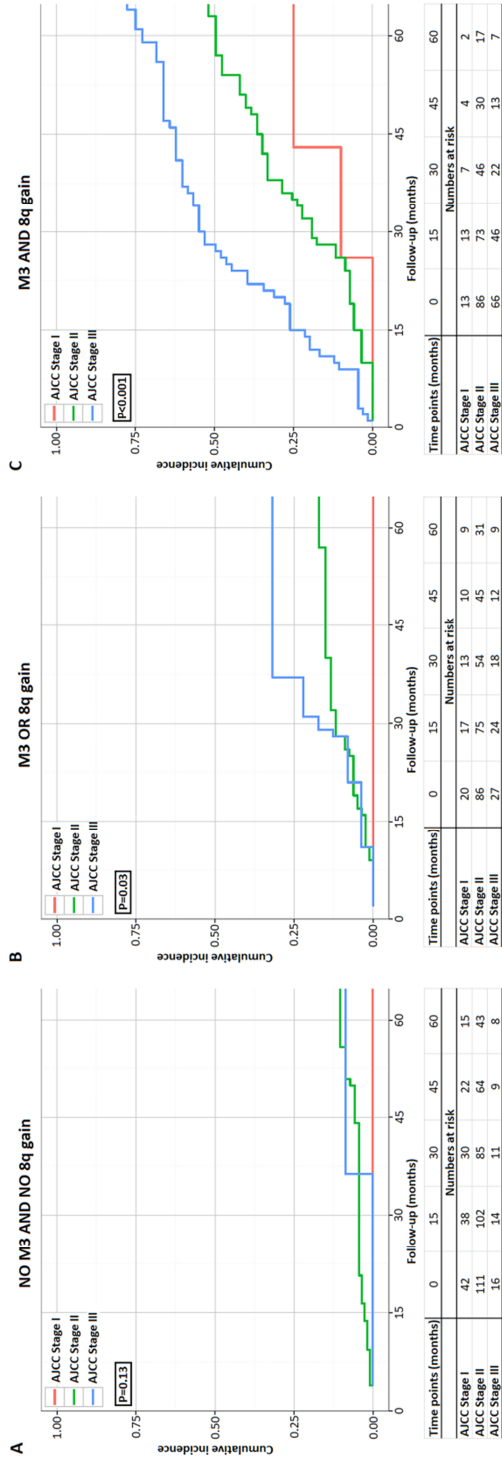
SUPPLEMENTS

Supplementary Table S1. Cox-regression model including gender, age at treatment, AJCC stage, and chromosome status. NS: non significant

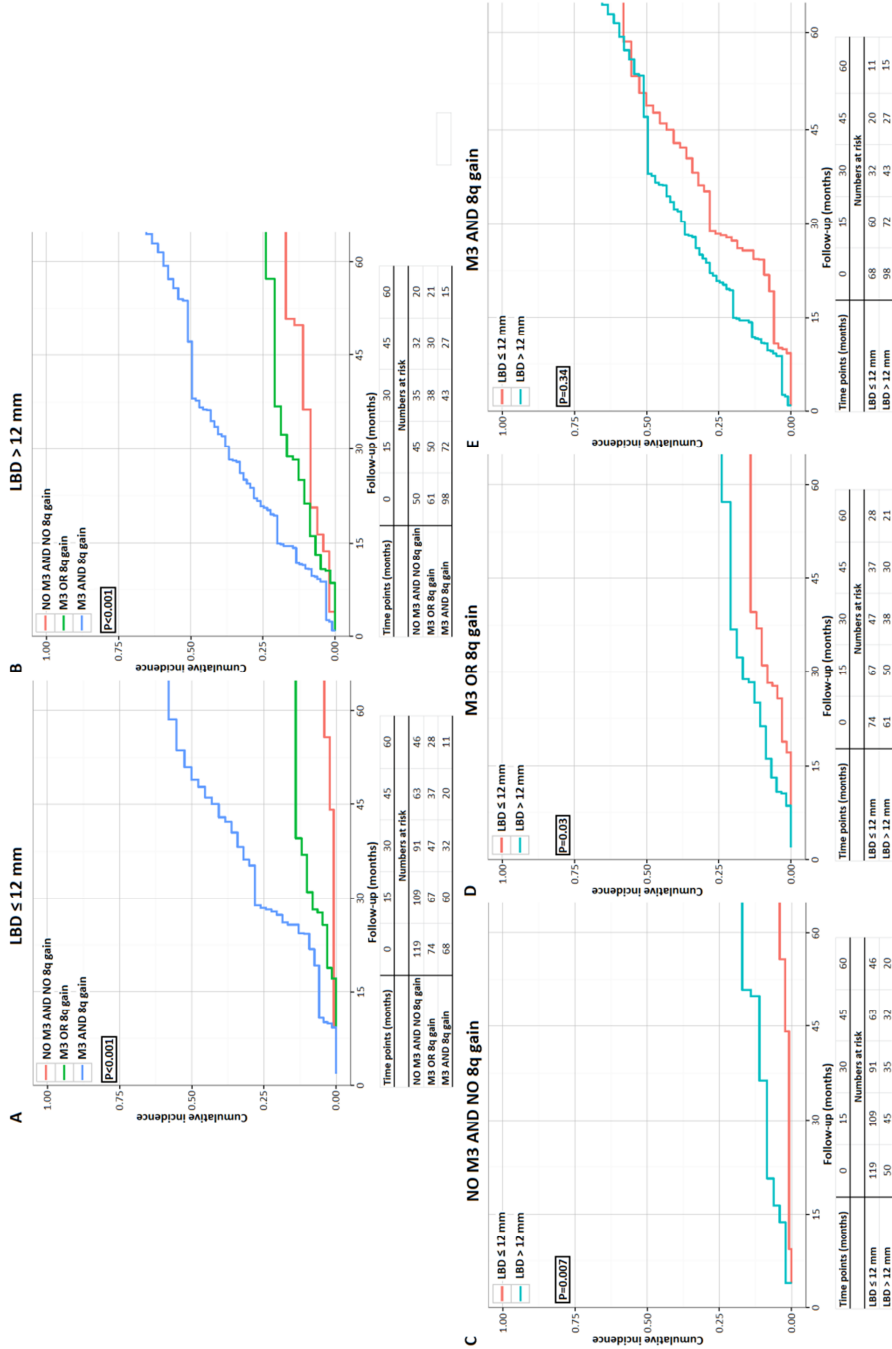
Characteristic	Univariate			Multivariable		
	HR	95% CI	P value	HR	95% CI	P value
Female gender	1.32	0.94 – 1.86	0.11			NS
Age at treatment	1.02	1.01 – 1.04	0.001			NS
AJCC Stage I <i>(reference category)</i>	-	-	-	-	-	-
AJCC Stage II	3.08	1.33 – 7.11	0.008	4.09	1.28 – 13.07	0.01
AJCC Stage III	10.08	4.35 – 23.35	<0.001	8.8	2.73 – 28.39	<0.001
No M3 AND No 8q gain <i>(reference category)</i>	-	-	-	-	-	-
M3 OR 8q gain	2.74	1.36 – 5.51	0.005	2.12	1.05 – 4.31	0.04
M3 AND 8q gain	11.71	6.37 – 21.53	<0.001	7.95	4.24 – 14.89	<0.001



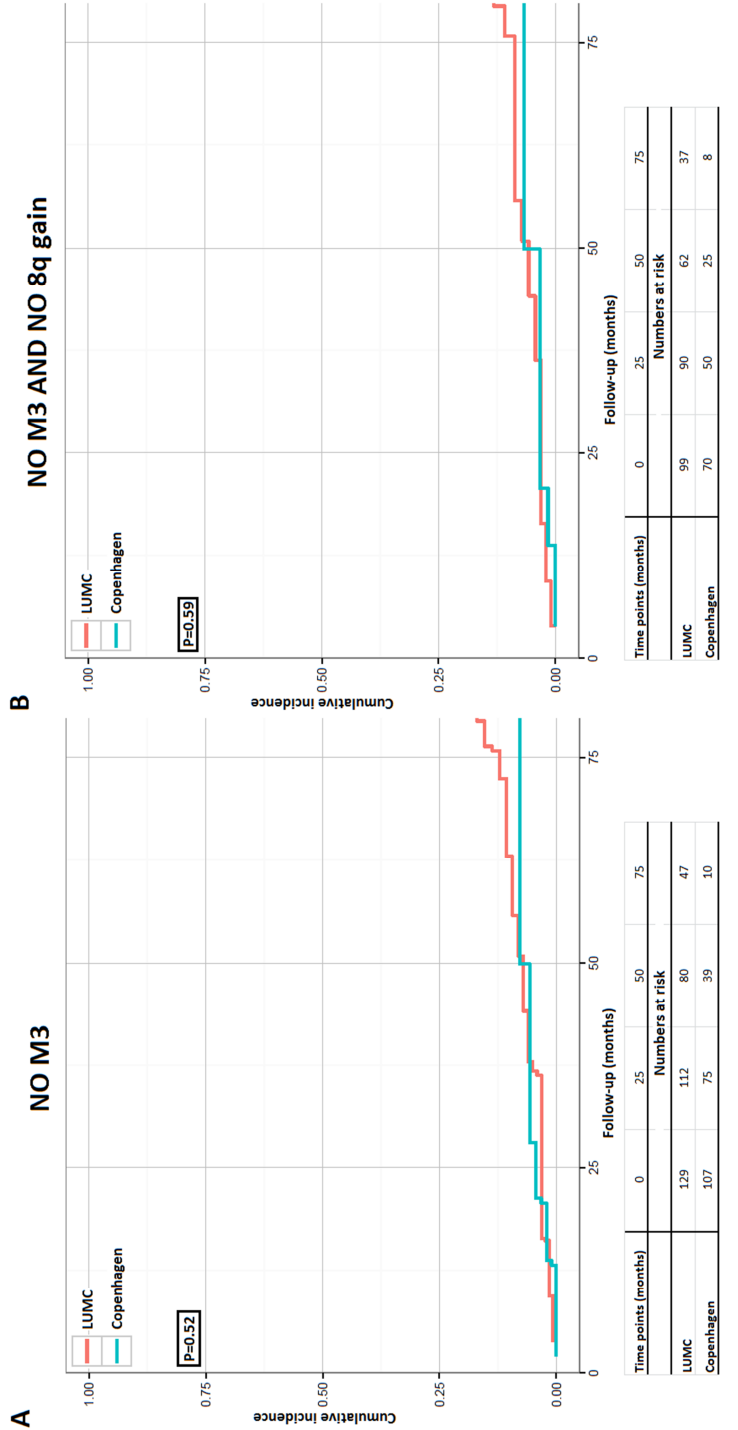
Supplementary Figure S1. Incidence of UM-related death in patients with only chromosome 8q gain or only monosomy 3 (M3).



Supplementary Figure S2. Incidence of UM-related death for the AJCC stages, analyzed in the different chromosome profile groups.



Supplementary Figure S3. Incidence of UM-related death when information on LBD (cut-off 12 mm) and chromosome 3 and 8q status is combined. First row: the effect of chromosome 3 and 8q status on incidence of UM-related death in tumors of different LBD. Second row: the prognostic effect of LBD in various chromosome profile groups.



Supplementary Figure S4. Comparison of the Danish and Dutch cohorts with regard to the incidence of UM-related death in tumors without monosomy 3 (A) and in cases with neither monosomy 3 nor chromosome 8q gain (B).

