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ORIGINAL ARTICLE

The Results of Stricter Inclusion Criteria in an Immunomagnetic Detection Study of Micrometastatic Cells in Bone Marrow of Uveal Melanoma Patients - Relevance for Dormancy

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Abstract Approximately 50% of uveal melanoma patients develop metastases. We want to evaluate the effect of stricter criteria on our data from our previous study correlating survival and bone marrow (BM) micrometastasis results using our immunomagnetic separation (IMS) method. Mononuclear cell fractions (MNC) isolated from BM were examined for tumour cells and the patients were classified as BM positive (BM+) or BM negative (BM-). The study originally included 328 consecutive patients with uveal melanoma from 1997 to 2006. The cohort was limited to 217 patients when we introduced cyto- or histopathological verification of melanoma cells in the patient as a main new criterion for inclusion. Tumour cells were found in BM-samples in 38.7% (95% CI, 32–45) at enrolment. Until the latest work-up 43.8% (95% CI, 38–50) of patients had developed melanoma metastases. After a minimum follow-up time of 8.5 years, 60.4% (95% CI, 54–66) of patients had died. The causes were: melanoma metastases 69.5%, another type of cancer 5.4% and non-cancerous causes 19.5%. Overall median survival was shorter for the BM- patients (11.3 years) (95% CI, 10–12) compared to the BM+ (16.5 years) (95% CI, 12–14), $p = 0.04$, log rank test. All-cause mortality and specific melanoma mortality estimated after 12 year follow-up showed a highly significant difference comparing BM- and BM+, $p = 0.010$ and p = 0,017, respectively. IMS yields a high fraction of BM+ samples due to micrometastasis at diagnosis and these cells appear to have a positive prognostic impact strengthening our previous report. The late recurrences support the concept of tumour dormancy.

Keywords Micrometastasis in bone marrow · Uveal melanoma · Exclusion criteria · Immunomagnetic detection · Survival · Tumour dormancy

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Introduction

Uveal melanoma is the most common primary intraocular malignant tumour in adults with the highest incidence being found in the Nordic countries [1]. More than 50% of patients with uveal melanoma develop metastases [2, 3]. Despite treating small melanomas with enucleation, subsequent metastatic dissemination occurs in some patients with T1a tumours. This old observation proves that early micrometastasis is a reality [4]. An experimental animal model documented that tumour cell dissemination occurs early [5].

Micrometastases may be present years before the tumour is diagnosed and treated, although revealing metastases at initial work-up occurs rarely. A substantial number of patients have subclinical metastases at presentation [6, 7]. The long interval from diagnosis, to symptoms of dissemination and death some months later, is a great puzzle. In uveal melanoma latencies of more than 40 years from diagnosis have been reported

[8]. Tumour dormancy, the latency of the metastatic process has been explained by tumour cell quiescence with a reversion to a non-proliferative state although key genes are regularly in the" on" position in melanoma cells [9]. The metastatic cells in bone marrow may be in a non-proliferative state [10]. Borthwick et al. [11] concluded that the most likely explanation was the inability of cells in metastatic sites to grow sufficiently large to require angiogenesis. No associated inflammation was observed which could ascribe inflammation as an explanation for this lack of growth. In uveal melanoma an inflammatory microenvironment is associated with BAP1 negativity (encoding BRCA1-associated protein 1) and poor outcome [12]. The turning point may be an escape from immune surveillance and control. The mechanisms that regulate the tumour cell antigenicity are unknown. Melanoma cells may influence dendritic cell (DC) functions, which are critical for the induction of anti-tumour immunity by inhibiting DC maturation, suppressing IL12 and IL10 production and increasing apoptosis [13].

Circulating tumour cells (CTCs) have been found in the blood of most cancer patients, also in uveal melanoma patients [14]. They are very rarely present in blood, and the number found is highly variable. It is therefore mostly of theoretical significance, with little use in clinical work. The hope is to use the reduction of tumour cells caused by treatment to monitor the effect of intervention. They could be a useful marker for residual disease to identify the patients who need adjuvant therapy. Treating micrometastatic disease is probably more effective than macrometastatic disease [15].

Our hypothesis at initiating the study was that CTCs and disseminated tumour cells (DTCs) are possible measures for improving prognostication in uveal melanoma patients. The main goal was to confirm that our earlier survival paradox also holds true for a more carefully selected subgroup and to discuss our findings in relation to dormancy.

Material and Methods

A prospective study was compiled from January 1997 to January 2006 at an ophthalmological referral centre in Norway. The Regional Committee for Medical Research Ethics, Southern Norway, approved the study (S-97072). Details about the material, the processing of BM and immunomagnetic detection techniques including sensitivity and validity and follow-up methods have been reported previously [6, 7]. In total 328 patients (162 males, 166 females) were included, for details see previous publications [6, 7]. Classification using the latest TNM system (7th Edition) is used in this report [16]. Photography of a BM+ case is shown to illustrate a positive patient. The medical details are described briefly (Fig.1).

To analyse the material more thoroughly, we decided to exclude confounding groups most likely to cause error in evaluating the BM-results in relation to mortality. After the exclusion process the remaining group of patients is named the Modified population sample.

We always strive for a diagnosis based on pathological tissue at diagnosis, dissemination and death. The main criterion for inclusion was the verification of melanoma cells in the patient with cyto- or histopathological documentation considered a necessity. Additionally, tumours accepted had to be located in the posterior segment. The date of diagnosis was defined as the day of the first BM procedure. All information used to include/exclude patients had to be available within the first year after diagnosis. Metastatic melanoma tissues obtained later were not sufficient. Patients dying of another cancer were not excluded.

Table 1 shows the reconsidered groups of patients, the Modified population sample. The reasons for exclusion of patients are detailed below:

1. Iris melanoma. The prognosis for iris melanomas <T3 is excellent [17] and in our material the only patient with iris melanoma with metastases probably had an incorrectly classified ciliary body melanoma with growth into the iris. Due to discrepancy in mortality, 32 patients with iris melanoma were excluded (10% of the total material).
2. Eye tumours (ETs) negative for 9.2.27, an antibody against the *NG2* gene [18]. We suppose that negative ETs also should have negative BM results. Ten patients tested negative in the primary tumour for the 9.2.27 anti-melanoma antibody. In two cases, samples from bone marrow tested positive with the same marker and were accepted. This result could be due to heterogeneity in ET, a phenomenon which has been documented in different tumour types including melanoma. Both patients are still alive. The remaining eight cases were excluded.
3. Possibility of another cancer [19]. Previous malignancy was not an exclusion criterion in the primary cohort. The tumour detected in the eye could be a metastasis from another cancer or an extra ocular melanoma. Twenty-three of these with previous cancer were accepted, 12 due to melanoma cells in the ET and 11 in the BM. On the other hand, 15 patients with another malignant disease within a period of ten years of inclusion in the study, starting nine years prior to or one year after the diagnosis of the malignant uveal melanoma, were excluded, lacking this confirmation. Cutaneous basalioma was not considered as an exclusion cancer.
4. Inconclusive histopathological melanoma diagnosis within one year of diagnosis. Fifty patients had no verification of melanoma cells during the first year after diagnosis i.e.

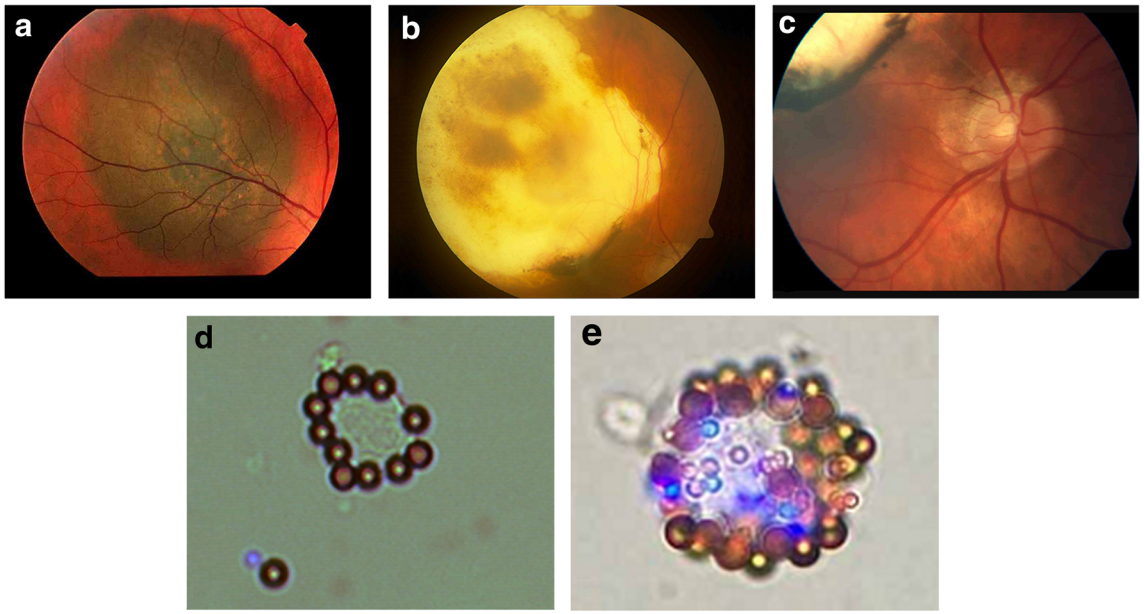


Fig. 1 Fundus photo of the right eye of an asymptomatic 57 year old man in 2002 with orange pigmentation but no peripheral retinal detachment, 9 months before treatment of a T1aN0M0 melanoma with a ruthenium plaque in 2003 after slight growth, supplemented with TTT 8 months later. (a) The eye was thereafter stable with visual acuity 0.2, photos 2009 and 2012 (**b**, **c**). Except for his melanoma he had always been healthy. A routine ultra sound of the liver revealed metastatic disease 125 months after treatment, verified with a liver biopsy and despite treatment with two different cytostatic systemic regimens, immunotherapy and antioestrogenic, he died 27 months later. A BM aspiration (sample number 240) at diagnosis in 2003 disclosed BM+ sample strongly positive (++) with 50 cells staining with two melanoma antibodies, 9.2.27 (a 250 kDa glycoprotein) and also with 376.96 (94 kDa glycoprotein). A routine retest 2 ½ year later in 2006 showed a slight positivity in BM with 9.2.27 (+) and unchanged for 376.96 (++).Illustration of an immunomagnetic positive BM sample with 9.2.27 (**d**, our routine staining) and a sample after triple staining with 376.96 (red latex) and EP-1(blue latex) (HMW melanom antigen) in addition (**e**)

no tumour cells in pathological specimens from biopsies of the primary tumour, enucleated eyes, metastases or tumour endoresection and all these cases were excluded. Verification of melanoma cells found in metastases or endoresection specimens performed more than one year after primary treatment were not accepted, 9 + 9 cases.

5. Cases not treated within one year of inclusion were excluded. This group includes a total of 6 patients, 3 BM+ and 3 BM-. The three BM+ are all alive, whereas one BM- has died of another cancer and two of melanoma dissemination.

Table 1 Number of patients excluded according to stricter criteria as shown

N = 328		Excluded
1	Iris tumour	32
2	Eye tumour NG2 negative (9.2.27 antibody)	8
3	Another cancer	15
4	Inconclusive FNAB/Enucl./Metastasis/BM negative	50
5	Observation only, or treatment delayed >1 year	6
Excluded		111

The reasons for the decisions are detailed in the table and material. The number of exclusions,111, in the Modified material are given. In all patients included, melanoma cells had to be verified the first year after diagnosis. In this way the original material of 328 patients was reduced to 217 in The Modified material

These modifications reduced the study population from 328 to 217 patients, 103 males and 114 females. The mean/median age at inclusion (the BM aspiration time) was 63.0/64.0 years (range 16–92).

Based on information from the most recent follow-up, we categorized the cause of death as due to: metastatic melanoma or to a non-melanoma related cause. Clinical and histopathological materials available at the time of death were routinely retrieved from clinical departments and general practitioners. Survival was measured from the time of entry into the study until death, or the date of latest observation. Patients still alive without melanoma metastases at the time of statistical work-up were treated as censored. Survival was estimated using the Kaplan-Meier method and Cox regression analysis and groups were compared with the log-rank test.

P-values <0.05 were considered statistically significant. Patient data collected until 1st of March 2015 were analysed with PASW 18 for Windows.

Results

Survival and BM Results

At enrolment, tumour cells in BM were detected in 84 (38.7%) 95% CI, 32–45) of the 217 patients. The survival of all patients classified according to test results as BM- or BM+ is shown in Fig. 2. Clearly, there is a better overall survival among the BM+ patients ($p = 0.04$) with a 50% median survival time of 16.5 years in the BM+ group versus 11.3 years in the BM- group.

Until the latest work-up, 95 (44%) (95% CI, 38–50) patients had developed melanoma metastases and 31 (33%) (95% CI, 23–41) of these tested BM+ at inclusion.

Forty patients have passed away of non-melanoma causes. Seventeen of these (42.5%) tested BM +. All seven patients dying of another cancer tested BM-. Some of the 33 others (7BM+/26 BM-, respectively) may possibly have been classified incorrectly. Only two autopsies were performed, both patients were BM+, but no metastatic disease was revealed.

In the original study, pathological confirmation of the eye diagnosis was done in 80% [6] with enucleation, FNAB or intraocular biopsy. At the time of the study outcome, in the Modified material sample, such confirmation had been obtained in 77/95 (81.1%) with disseminated melanoma, and in 7/7 (100%) with another cancer and only 6/33 (18.2%) with a non-cancer death. In total, 90 of 131 were pathologically verified (69.0%). In addition an oncologist confirmed the diagnoses of dissemination without tissue samples in 16 (16.9%) and in one single case this information was lacking.

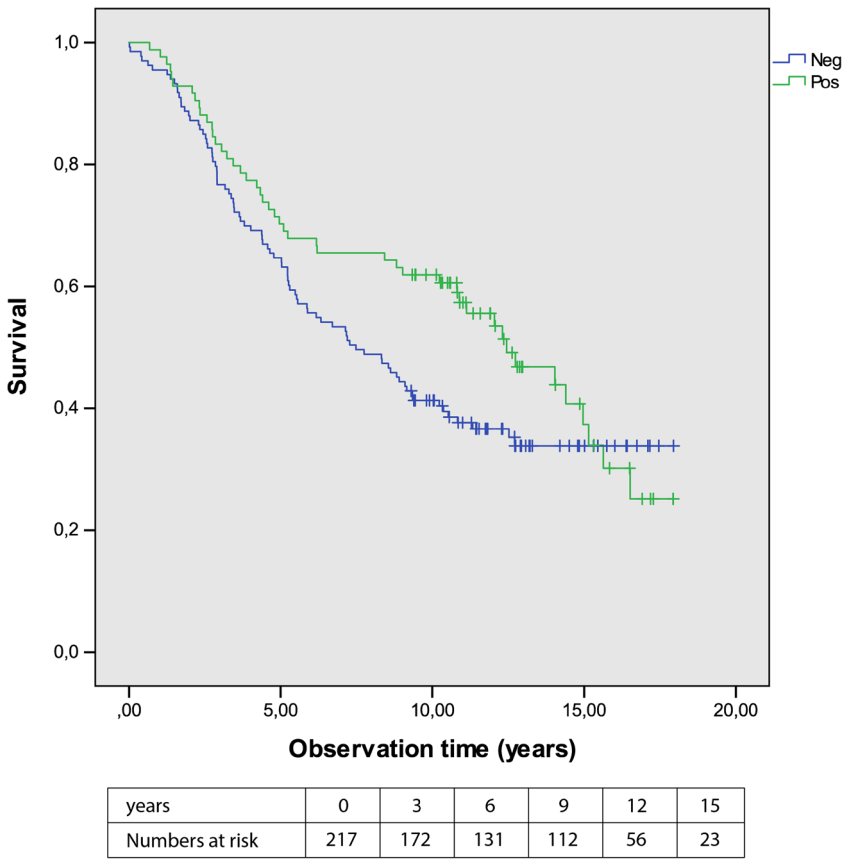
The all-cause mortality was calculated from 131 cases of 211 (60.4%); after five years (71, 32.7%), ten years (110, 50.7%), fifteen years (128, 59%), see Fig. 2.

Melanoma specific mortality was calculated from 91 cases of 211, 41.9%; after five years (51; 23.5%), ten years (78; 35.9%), and fifteen years (88; 40.6%) at the same intervals, see Fig. 3.

At the time of study closure, 86 patients were still alive, 40 BM+ and 46 BM-, compared to 84 BM+ and 133 BM- at beginning of the study, changing the ratio from 0.63 to 0.87.

Discussion

This study focuses on the relationship between the presence of micrometastases in BM at diagnosis of uveal melanoma and patient survival in the long-term. We recently reported an unexpected association between lack of melanoma cells in BM at inclusion and poorer prognosis compared to those with melanoma cells. Here we perform a survival analysis of an updated dataset and show that with stricter inclusion criteria and longer follow-up the findings in our previous



years	0	3	6	9	12	15
Numbers at risk	217	172	131	112	56	23

Fig. 2 Overall survival is significant higher for the BM positives (84) (green line) at first sampling at inclusion in the study as compared to the BM negative patients (133) (blue line) ($p = 0.04$, log rank test. The all-cause mortality is used. Vertical marks: Censored patients (55 positive, 71 negative). Ticks show censored observations and numbers below graph represent patients at risk

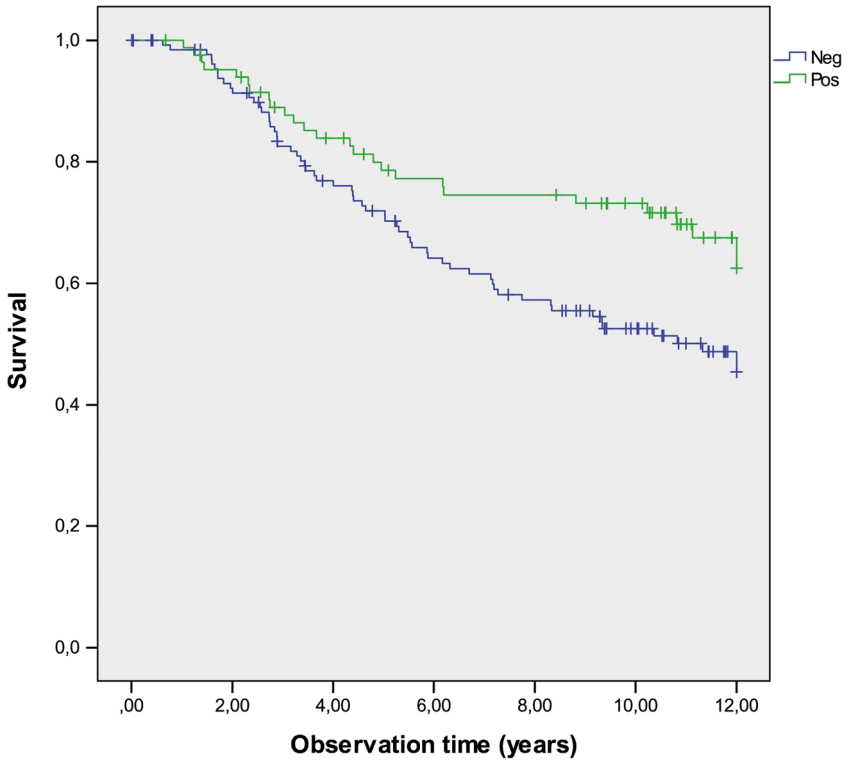


Fig. 3 The melanoma specific survival is significant higher for the BM positives (84) (green line) at first sampling at inclusion in the study as compared to BM negative patients (133) (blue line) ($p = 0.017$, log rank test). The curves are changing direction and are approximating paralellisme about 13–14 years. We therefore cut the curves at 12 years to do a Cox regression analyse in the modified material with all patients cencored after 12 years. Vertical marks: Censored patients (58 positive, 71 negative)

article [7] are confirmed and strengthened**.** Thus the counter-intuitive result, that tumour cells present in BM aspirates detected with 9.2.27 antibody at the time of diagnosis indicate a better prognosis, is substantiated.

The survival time in our material after five, ten and fifteen years is comparable to other studies. The total mortality is higher for BM- than for the BM+ patients from the third year, with a maximum at eight, and lasting until the 15th year has passed (Fig. 2). The difference in specific melanoma mortality between BM+ and BM- patients is highly significant (Fig. 3). In the modified population sample, with all patients censored at 12 years, the significance was 0.017 (Kaplan-Meier) for melanoma specific mortality, compared to 0.010 for all-cause mortality. This significance is maintained after sex and age is taken into the Cox regression analysis.

After a 15 year observation time, the mortality seems to be increasing in the BM+ group and stable in the BM- group (Fig. 2). The most probable explanation for this phenomenon is that in the latter group, the relative number with a true localized disease [20] increases with time, creating a curve almost parallel to the abscise, in contrast to the BM+ that in our opinion has dissemination outside the eye at diagnosis and therefore encompasses a potential for metastatic death as long as the BM+ group exists. The bone marrow is supposed to be a filter or a homing organ for malignant cells. These dormant cells are difficult to detect. With PECT/CT scan [21] the BM is the second most frequently affected organ in melanoma dissemination.

Different methods for identification of micrometastatic melanoma cells have been used, all with inherent limitations. Isolation of CTCs and DTCs relies on the specific and consistent expression on tumour cells, as well as on the method used for detection. In 29 healthy volunteers in a melanoma study such cells were not detected with the same method [22]. All tumour cells were evaluated by microscopy and tumour cells showed abnormal size and high nuclear/cytoplasm ratio [23]. In these circulating melanoma cells Ulmer et al. [24] frequently found monosomy 3. The tumour cells were often identified with 3 different surface melanoma markers and sometimes with immunocytochemistry using HMB45 [6, 23]. Moreover, Tura et al. [25] reported that melanoma cells detected were cultured from CTCs showing that most of the cells are viable. Uveal melanoma cells have also been cultured from our cohort, but not systematically.

At diagnosis of the eye tumour, our study detected extraocular melanoma cells in BM in 30% of the patients [6, 7] and in the modified material the result was 38.7%. We routinely used only one antibody, 9.2.29, targeting the melanoma-associated chondroitin sulphate proteoglycan (MCSP), named also neural/glial antigen 2 (*NG2*). Freeman et al. [26] reported that in a limited study using immunomagnetic enrichment on blood samples from cutaneous melanoma patients, a combination of markers gave a better yield and higher number of enriched cells with a combination of antibody-coated beads compared to a single antibody ($p = 0.007$). In another study, [27] performed experiments with five human uveal cell-lines and three different antibodies, NKI/C3, NKI/beteb or 9.2.27.

Their spiking experiments showed that a combination of the three antibodies was highly efficient, and that the 9.2.27 antibody, if used alone, was insufficient to capture the cell line SP6.5 in blood below a certain concentration. They speculated that this fact can be a proof of heterogeneity in the tumour cell population and that a multi-marker approach allows detection of cells with different phenotypes. The clinical part of their study showed that nearly all uveal melanoma patients will test positive for CTCs if an adequate panel of antibodies is used. However, spiking experiments with cell lines may not necessarily reflect the situation with fresh patient samples. Moreover, their antibody panel included an antibody binding to NKI/C3, which is CD63, a tetraspanin and an endosomal and exosomal marker expressed on both hematopoietic and non-hematopoietic cells.

Nested reverse-transcriptase PCR detection was used by Callejo et al. [28] who found circulating tumour cells in 29 of 30 uveal melanoma patients without known metastasis over two years with multiple testing, and in more than 85% of the patients visits, but with a low cell count (<4 cells) and in few of the nested samples (<4 of 10 samples). An excess of CTCs seemed not to be a limiting step in the metastatic process. In our material [7] there was no correlation between the number of cells detected in the samples and the risk of dissemination. Suesskind et al. [14] did not see any changes in CTCs before or after different treatment modalities and there were no significant association to prognostic factors.

In general, the low number of micrometastatic tumour cells detected makes it difficult to determine if an observed increase/decrease is significant. The cell shedding is presumably of a discontinuous nature and the tumour cells are likely to undergo some molecular changes in the circulation [25]. Surprisingly, the last authors reported a yield of 93.5% from blood samples in 31 patients with primary nonmetastatic UM using a dual-marker immunoenrichment with NKI/beteb and NKI/C3 antibodies. Moreover, melanoma cells were detected in their cultures from CTCs samples in 15 of 29 patients, documenting viable cells. Additionally, they documented monosomy-3, which is significantly associated with an advanced tumour stage [29] in CTC in 58% of the patients with a novel immune-FISH assay**.**

Interestingly, a bimodal mortality dynamic for uveal melanoma has been found in three explored hazard rate curves from older melanoma materials [30]**.** This bimodal pattern of mortality has been reported in other malignancies, including breast cancer, and cannot be explained by a continuous growth pattern. The curves support a model where growth is interrupted with episodes of tumour dormancy. This is in agreement with the long latency without symptoms, followed by a short interval with symptoms before death occurs, as seen both in the study of Zimmerman [31] and in our BM melanoma studies [6, 7]. A second mortality peak for uveal melanoma was suggested from the data of Packard [32]. Demicheli et al. [30] found bimodal mortality in this material of enucleated patients as well as patients treated with radiation therapy with different time spans and at different institutions collected from three previous studies. The first mortality peak is in the second postoperative year. The second peak was believed to be a consequence of a rapid growth of dormant tumour cells in a reservoir after interrupted dormancy [30].

Damato [20] speculated that uveal melanomas may behave in a diverse manner, i.e. that some disseminated early even though overt metastases are not detectable, similar to our BM+ patients. Others develop metastatic capability gradually after the tumour is detected, and in this group delayed treatment can have catastrophic consequences. A third group is without dissemination and has local disease only, and is always truly BM-. There is evidence in favour of this view, but there is no knowledge about the relative frequencies of the three possibilities. Some of the eye tumour 9.2.27 positive patients who tested BM- could belong to this third group, especially when repeated BM testing was negative. However, our material shows shorter survival of the BM- patients, indicating that these "true BM-" at best represent a small portion of this group. Most "false BM-" probably must have micrometastatic melanoma cells not present or not found in our BM testing to explain the curves in Fig. 2. Our antibody did not detect all the surface melanoma epitopes [25–27]. Interestingly, manipulation of the melanoma cell surface with detergents in 10 large melanomas did not change our yield of BM+ samples (tried after the closure of the study, data not shown). With immunocytochemistry directly toward the mononuclear BM fraction, however, we found a few patients in the study population including one bilateral case, positive for HMB45, an intracellular epitope at diagnosis [6].

We think that our antibody catches an epitope that is downregulated or lost over time in some of the BM- patients in a more advanced stage with larger tumours. This phenomenon has been shown for lymphoma where the epitope pattern varies throughout the stages of illness, in part induced by the applied therapy. The clinical pictures and the prognosis fluctuated with this variation [33]. Our antibody may therefore select the melanoma with a better prognosis than the average in the cohort, although we don't see a late top. The second top described by Demicheli et al. [30] can be caused by phenomena like this**.** In an albino rabbit model, transcriptional profiling of uveal melanoma cells from human cell lines were tested in specimens from the primary tumour, the circulating cells (CTCs) and the metastases in the lungs (DTCs). The study showed that the specific melanoma marker MelanA substantially decreased in expression from the original tumour to metastatic tissue. Dedifferentiation resulted in downregulation of the melanoma-specific marker MelanA [34].

Tumour dormancy is a clinical phenomenon where the tumour cells are present but remain asymptomatic and undetectable over a prolonged period of time. The disseminated

tumour cells remain dormant in their new microenvironment until the conditions become favourable for their growth**.** In our opinion the cohort of dormant cells are a mix of both BM+ and BM- melanoma cells. Both groups have some cases with survival for more than 10 years. No signature markers of dormancy have been well documented. The marker- 9.2.27-reveals uveal melanoma cells homing in BM at diagnosis in more than 30% by our investigations [7]. Homing in BM is observed also for small uveal melanoma after complete tumour eradication**.** The mechanism of the switch from the dormant to the proliferative state that initiates tumour growth and mass expansion is unclear, and the length of melanoma cell dormancy in micrometastasis is unpredictable. The inadvertent transmission of melanoma from a patient who served as an organ donor following sudden brain death, is illustrating. The donor had been treated for a melanoma 16 years prior to his death and was living without symptoms, signs or detectable metastases and presumed to be healthy. Two immunosuppressed organ recipients died of melanoma metastasis two years later [35], demonstrating the existence of dormant melanoma cells in the transplanted organs.

Micrometastasis may be established years before the eye melanoma is diagnosed. In a mouse model Eyles et al. [5] showed that tumour cell dissemination occurred weeks after the clinical onset of the primary tumour, but immunosurveillance limited metastatic overgrowth. Detectable metastatic lesions became apparent up to 1.5 years later. The genomes of the primary and secondary tumours were similar.

Several limitations in our study should be considered. Firstly, the exclusions are performed retrospectively. It is not unusual to study subsets of a larger material in isolation if the subset is selected using criteria that have been defined ahead of the outcome, as in this study. The time span makes it impossible for us to do a confirmation study with a second group of patients rather than reanalyse our data.

Secondly, the number of exclusions is high, reducing the material to 2/3 of the original cohort. Finally, we included patients with a positive BM test and a posterior melanoma, accepting at least two melanoma cells in BM as evidence of a uveal melanoma. We have been consistent in using our previously specified criterion for a positive bone marrow sample - a presence of at least two rosettes, i.e. two different melanoma cells with more than five magnetic beads surrounding each of them. In our first article on the survival paradox [7] the group of patients with only one rosette were considered BM negative, despite having a medically ambiguous test result. This group was meaningfully different from the BM negative patients, and the group was documented to have the second highest mortality in the material as a whole. This suggests that finding even a single melanoma cell can be of clinical importance**.**

However, the modified study population contains over 200 patients, the follow-up is long and the documentation of pathology in the eyes and metastases is good. The exclusion criteria are based on melanoma biology. All patients included had a verification of melanoma cells to make sure that no one is included in the material without their diagnosis of melanoma being pathologically verified. This is unusually stringent in studies of melanoma. Our results and the recent scientific data on bimodal mortality and tumour cell dormancy may reflect that late metastasis is part of the biology of uveal melanoma. Patients who survive more than ten years without metastases after treatment cannot be considered cured [36]. The expression of NG2 staining with the 9.2.27 antibody has been associated with an enhanced malignant potential in vivo and in cell culture with increased proliferation of melanoma cells [18, 37]. Our results document a different, reduced aggressiveness in BM+ uveal melanoma.

In conclusion, the cells detected in BM in 39% at diagnosis are mostly vital melanoma cells, documenting that dissemination is an early manifestation in uveal melanomas. The melanoma antibody used probably selects only a fraction of these dormant melanoma cells that are homing in bone marrow, a subgroup with a positive impact on survival. This article is a continuation of our other four articles on micrometastatic spread from uveal melanomas [6, 7, 23, 38] and gives a thorough discussion of micrometastasis.

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