Microvascular Density in Predicting Survival of Patients with Choroidal and Ciliary Body Melanoma

Teemu Mäkitie, Paula Summanen, Abti Tarkkanen, and Tero Kivela

PURPOSE. Although malignant uveal melanoma disseminates predominantly hematogenously because of the absence of intraocular lymphatics, consensus about prognostic impact of microvascular density (MVD) has not been reached. This study was undertaken to investigate whether MVD, microvascular patterns, or both determine prognosis of uveal melanoma.

METHODS. A population-based retrospective cohort study of melanoma-specific and all-cause mortality of 167 consecutive patients who had an eye enucleated because of choroidal or ciliary body melanoma from 1972 through 1981 was conducted. MVD was determined by counting tumor vessels in a masked fashion from areas of highest vessel density after immunostaining for CD34 epitope, factor VIII-related antigen (FVIII-RAg), and α-smooth muscle actin (SMA). Kaplan–Meier and Cox regression analyses of survival were performed. The association between MVD and tumor size and location, cell type, and microvascular patterns was assessed.

RESULTS. MVD could be determined from 154 of 167 melanomas (80%). Based on globally highest count obtained with antibodies to CD34, MVD ranged from 5 to 121 vessels/0.313 mm² (median, 40) and its association with presence of microvascular loops and networks (P = 0.0006), epithelioid cells (P = 0.028), and largest basal tumor diameter (P = 0.0029) was statistically significant. The 10-year melanoma-specific mortality increased with MVD (0.09, 0.29, 0.59, and 0.64, according to quartiles; P < 0.0001), as did all-cause mortality (P = 0.0022). Equivalent results were obtained with immunostaining for FVIII-RAg, whereas MVD obtained with antibodies to SMA was not associated with prognosis. Cox regression showed a hazard ratio of 2.45 (95% CI, 1.43–4.18) for presence of epithelioid cells, 1.11 (95% CI, 1.03–1.20) for largest basal diameter, 1.23 (95% CI, 1.06–1.43) for square root-transformed MVD, and 1.51 (95% CI, 1.09–2.10) for presence of loops and networks, all of which independently contributed to prognosis.

CONCLUSIONS. The findings support the theory that both MVD and microvascular patterns contribute independently to prognosis in uveal melanoma in addition to cell type and size of the tumor. (Invest Ophthalmol Vis Sci. 1999;40:2471–2480)

The growth, progression, and metastasis of cancer depend on adequate blood supply and therefore on vascularization. High microvascular density (MVD), a widely applied morphologic measure of vascularization, independently predicts death from several types of cancer, including tumors that frequently spread through the lymphatic route, such as breast cancer. A significant minority of studies on MVD have failed to document this relationship, however. Malignant melanoma of the uvea is the most common intraocular cancer in humans. Because no lymphatic vessels emanate from the eye, the cancer can only disseminate hematogenously, unless it shows extraocular extension with invasion of conjunctival lymphatics. Not surprisingly, the prognostic significance of microvessels in uveal melanoma has been evaluated in a number of recent reports that have included quantitative variables such as MVD and qualitative ones such as microvascular patterns that are used to assess the arrangement of microvessels.

The concept of microvascular patterns was introduced by Folberg et al., who suggested that microvessel architecture has a stronger association with prognosis than previous clinical and histopathologic prognostic indicators, including largest basal tumor diameter, ciliary body involvement, and presence of epithelioid cells. We have provided evidence in favor of their theory by showing that microvascular loops and networks independently predicted tumor death in a consecutive, population-based series of choroidal and ciliary body melanoma.

Although microvascular patterns also have usually, but not always, been associated with melanoma-related deaths in other data sets, a major disagreement exists about the prognostic impact of MVD in uveal melanoma. Of three studies published so far, two reported a negative association. However, based on a data set of 116 patients, who had a disproportional number of tumors that metastasized, Foss et al. suggested that MVD may predict fatal outcome of uveal melanoma. Moreover, they did not find an independent relationship between microvascular patterns and survival after adjusting for MVD. The finding that MVD was
higher in tumors with microvascular loops and networks suggested to them that the effect on prognosis of microvascular patterns may be secondary to high MVD, a factor that was not analyzed by Folberg et al.12,19,23

We designed and conducted a study to resolve whether quantifying MVD in uveal melanoma helps to predict the prognosis and to what extent MVD and microvascular patterns are interrelated.

**Patients and Methods**

**Study Design**

Our primary purpose was to confirm or disapprove an independent association between MVD and survival of patients with malignant uveal melanoma. Our secondary goal was to analyze the relationship between MVD and microvascular loops and networks. A third goal was to compare the prognostic significance of microvessel counts obtained with two different endothelial markers and two methods of counting microvessels in uveal melanoma. Our institutional review board approved the study, and the tenets of the Declaration of Helsinki were followed.

Calculation of the number of patients needed to show a statistically significant difference between MVD and survival was by simulation,24 which was based on analysis of MVD in 116 cases of malignant uveal melanoma by Foss et al.8 who published Kaplan–Meier curves for patients divided into four quartiles based on the highest vessel count. The estimated 9-year cumulative probabilities of survival for the four quartiles were 0.85, 0.55, 0.44, and 0.27.8 Averaging the first two and last two quartiles, we estimated the 9-year probabilities of survival of patients with a lower and higher MVD than the median to be 0.70 and 0.35, respectively, with a 0.35 difference of all malignant uveal melanomas treated during that time.

**Study Population and Exclusion Criteria**

Briefly, the 167 consecutive patients, all of whom had had choroidal or ciliary body melanoma enucleated between 1972 and 1981, were ascertained from diaries of the Ophthalmic Pathology Laboratory, Helsinki University Central Hospital.20 All but the smallest uveal melanomas, and all eyes enucleated in our district were submitted to the Ophthalmic Pathology Laboratory, Helsinki University Central Hospital.20 Because the number of evaluable specimens was low (85% necrotic), we included all but the smallest uveal melanomas and all eyes enucleated in our district were submitted to the Ophthalmic Pathology Laboratory, Helsinki University Central Hospital.20

**Assessment of MVD**

The paraffin blocks were cut at 5 μm, and thereafter the slides were randomly coded by an outside laboratory technician. The code was broken only after MVD and survival data were ready for analysis, with all investigators masked to the outcome of individual patients until that time.

Immunostaining of microvessels was performed using the avidin–biotinylated peroxidase complex method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA) as described previously in detail.20 The primary mouse monoclonal antibody (mAb) QBEND/10 (diluted 1:25; lot 121202; Novocastra Laboratories, Newcastle-upon-Tyne, UK) to the CD34 epitope of the endothelial cells used in the study immunostains vascular endothelia effectively in paraffin sections.27,28 Vascular endothelial cells were also identified with polyclonal rabbit antibodies to FVIII-Rag (1:400; Dakopatts, Copenhagen, Denmark). For comparison, microvessels with a muscular layer were immunostained with mouse mAb IA4 (1:8000; lot 98F4808; Sigma, St. Louis, MO) against α-smooth muscle actin.29 Ciliary muscle acted as an internal positive control.

To enable evaluation of immunoreaction in pigmented tumors, the peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride and, regardless of the grade of pigmentation, melanin was then bleached with 3.0% (vol/vol) hydrogen peroxide and 1.0% (wt/vol) disodium hydrogen phosphate, as described previously.20 This sequence obviated any problems of altered antigenicity that may occur if bleaching is performed before immunostaining. All immunostainings with mAb QBEND/10 were satisfactory. One specimen stained for FVIII-Rag, and four specimens stained with mAb IA4 were repeatedly unsatisfactory.

Microvessels were counted from three separate, most highly vascularized areas (“hot spots”) according to Foss et al.7,8 The three areas were identified by scanning the entire immunostained tumor at ×100 magnification. Vessels were then counted at ×200 magnification using an eyepiece with an etched square graticule (WK 10x/20L-H; Olympus, Tokyo, Japan). The area of the graticule was 0.313 mm², measured with an object micrometer (Ernst Leitz, Wetzlar, Germany).

Any immunolabeled vessel, clearly separate from an adjacent one and either totally inside the graticule or touching its top or left border, was counted as a microvessel.4,7,8 To assess intraobserver reproducibility, hot spots were reidentified in a masked fashion 6 months later from a subset of 31 slides immunostained with mAb QBEND/10, chosen on the basis of a random-number table.
Assessment of Microvascular Patterns

Microvascular loops and networks, consisting of at least three back-to-back loops, were identified according to the criteria of Folberg et al.12,19 from sections first bleached with potassium permanganate and oxalic acid and then stained with periodic acid–Schiff without counterstain, as described previously in detail.20 The sections were viewed under a green filter (Wratten No. 58, Eastman Kodak, Rochester, NY).

Statistical Analysis

All statistical analyses were performed with a statistical software program (PC-90; BMDP Statistical Software, Cork, Ireland).

For analysis of MVD, globally highest counts and the mean of the three highest counts were alternatively used and compared. The deviation of all counts from normal distribution was statistically significant, when evaluated by the Shapiro–Wilk test ($P < 0.0001$ for all counts). Normal distribution was approximated after square root transformation of the counts (range of $P$ from 0.097–0.19), except for the mean of the three highest counts of FVIII-RAg–labeled and the globally highest counts of $\alpha$-smooth muscle actin–labeled vessels ($P = 0.040$ and 0.029, respectively).

Agreement between microvessel counts obtained from sections labeled with antibodies to the CD34 epitope and FVIII-RAg was assessed by plotting the difference between the two counts against their mean and by calculating the mean difference with 95% limits of agreement.31 Because the difference increased with increasing counts, the comparison was based on square root–transformed counts.32 Intraobserver reproducibility was assessed similarly.

To compare MVD in various types of uveal melanoma, untransformed counts between two and more groups were compared with the nonparametric Mann–Whitney test and Kruskal–Wallis test.31 In addition, square root–transformed MVD was compared with Student’s $t$-test and one-way analysis of variance.31

Univariate analysis of melanoma-specific survival was based on the Kaplan–Meier product-limit method, and survival curves were compared with the Mantel–Cox test.25 Patients judged to have died of other causes were censored at their time of death. To guard for the possibility that they were more or less likely to have progression of melanoma than other patients, all-cause mortality was also analyzed. Equality of follow-up between groups was ascertained by comparing Kaplan–Meier curves with reverse censoring.25

For Kaplan–Meier analysis, the series was divided into quartiles based on MVD.8 The cell type was collapsed into two categories based on the presence of epithelioid cells (spindle versus nonspindle),7,12,26 and tumor location was dichotomized according to the presence of ciliary body involvement. Largest basal tumor diameter was divided in three categories: small ($\leq 10 \text{ mm}$), medium ($>10–15 \text{ mm}$), and large ($>15 \text{ mm}$).12 The effect of microvascular loops and networks was analyzed by using a combined categorical variable that considered networks to be an advanced stage of loops (no loops, loops without networks, and networks).20

Multivariate analysis of survival was based on the Cox proportional hazards model.25,55 MVD was analyzed as a continuous variable using square root–transformed counts. The best model previously obtained for this group of patients was used as a starting point20 and adjusted for MVD. The regression coefficients and hazard ratios (HRs) with 95% confidence intervals were calculated. The assumption of proportional hazards was ascertained by complementary log plots.25 Appropriateness of the model was confirmed by forward and backward stepwise regression. The best model obtained by Foss et al.8 in their data set was also fitted, after adjusting counts with a factor of 0.8 to account for the slightly different area they used for counting microvessels (0.250 mm$^2$ versus 0.313 mm$^2$).

Possible interaction between MVD and microvascular patterns, cell type, and largest basal tumor diameter was tested by comparing the main model with models that included product terms involving these variables.55

RESULTS

Microvessels as a rule were more distinct and easier to count from sections immunolabeled for CD34 epitope, compared with sections labeled for FVIII-RAg (Figs. 1A through 1D). Both methods revealed vessels also in areas where periodic acid–Schiff–stained microvascular patterns were either indistinct or seemingly absent (Figs. 1E, 1F). They outlined microvascular loops and networks when these were present (Figs. 1G, 1H, 1I).

MVD

MVD varied widely from melanoma to melanoma. The median MVD based on the globally highest count was 40 vessels/0.313 mm$^2$ (range, 5–121), and the median MVD based on the three highest counts averaged was 33 vessels/0.313 mm$^2$ (range, 5–113), analyzed with antibodies to the CD34 epitope (Figs. 1A, 1C). The corresponding MVDs obtained with antibodies to FVIII-RAg were 33 (range, 6–102) and 27 (range, 4–84) vessels/0.313 mm$^2$ (Figs. 1B, 1D, respectively).

The mean difference between MVD obtained with antibodies to the CD34 epitope and FVIII-RAg, evaluated by square root–transformed, globally highest counts, was 0.50 units (95% CI, 0.37–0.65) if favor of antibodies to the CD34 epitope, corresponding to a median difference of four vessels per counted area (Fig. 2A). For the square root–transformed three highest counts averaged, the difference was 0.42 units (95% CI, 0.37–0.65), corresponding to 3.5 vessels per counted area.

Antibodies to $\alpha$-smooth muscle actin immunolabeled precapillary arterioles. The median MVD based on the globally highest count was six vessels/0.313 mm$^2$ (range, 1–24), and the median MVD based on the three highest counts averaged was four vessels/0.313 mm$^2$ (range, 1–17).

Intraobserver reproducibility in assessing MVD with antibodies to the CD34 epitope, evaluated by the difference between initial and repeated square root–transformed counts from reidentified hot spots, was 0.28 units less (95% CI, 0.07–0.50) after recounting on the basis of the globally highest count (Fig. 2B), and 0.25 units less (95% CI, 0.02–0.48) after recounting on the basis of the three highest counts averaged, corresponding to 3 and 1.7 vessels less per recounted area, respectively.

Association with Other Prognostic Variables

MVD obtained with antibodies to CD34 and FVIII-RAg were significantly higher in tumors that contained microvascular loops and networks than in tumors without them (Table 1). Overlap between tumors with and without loops and networks
was pronounced, however (Fig. 3A). Consequently, high MVD could be obtained from melanomas that had no loops and networks (Figs. 1E, 1F), and in melanomas with these patterns, the most densely vascularized areas seldom coincided with areas of loops or networks (Figs. 1G, 1H, 1I). This was not only because vessels forming loops run for a longer than average distance in the same plane of section but also because other microvessels were generally excluded from within individual loops (Figs. 1G, 1H).

The MVD was significantly higher also in melanomas that contained epithelioid cells (Fig. 3B, Table 1), and a weak positive statistically significant correlation was noted between MVD, largest basal tumor diameter ($P = 0.002$, linear regression), and tumor height ($P = 0.0004$; Fig. 3C, D). Substantial overlap was again observed, especially in tumor size (Figs. 3B, 3C, 3D). MVD was not associated with ciliary body involvement, extraocular extension, sex, and age of the patient (Table 1). Similar results were obtained by analyzing square root-transformed vessel counts with parametric methods (Table 1). Microvessels identified with antibodies to $\alpha$-smooth muscle actin were equally common among all these groups (Table 1).

**Univariate Analysis of Survival**

At the end of the follow-up, 37 of 134 patients (28%) were alive without evidence of recurrent melanoma, 59 (44%) had died of metastatic uveal melanoma, 37 (28%) had died of other causes, and 1 (1%) had been lost to follow-up.

As analyzed by the globally highest MVD obtained with antibodies to the CD34 epitope, the 10-year cumulative
melanoma-specific probability of survival decreased when the MVD increased (0.91, 0.71, 0.41, and 0.34 for the four quartiles from lowest to highest density; Fig. 4A; \( P < 0.0001 \), log rank test). The difference was very similar when the mean of the three highest vessel counts was analyzed (Fig. 4B; \( P < 0.0001 \)) and when these comparisons were based on counts obtained with antibodies to FVIII-RAg instead (Figs. 4C, 4D; \( P = 0.0022 \) and \( P < 0.0001 \), respectively). In contrast, the 10-year cumulative melanoma-specific probabilities of survival were not associated with MVD obtained with antibodies to \( \alpha \)-smooth muscle actin (Figs. 4E, 4F; \( P = 0.41 \) and 0.61, respectively).

The single threshold count that most efficiently separated patients with low and high risk of melanoma-caused death was 39 vessels/0.313 mm\(^2\) as analyzed by antibodies to the CD34 epitope (10-year survival, 0.82 versus 0.38; 20-year survival, 0.70 versus 0.27; \( P < 0.0001 \)). None of the 16 patients who had MVD counts of 15 or less had died of melanoma.

Analysis of all-cause mortality instead of melanoma-specific mortality produced equivalent results in all six comparisons (Fig. 4G; Table 2). The presence of microvascular loops and networks in this data set was strongly associated with melanoma-specific (Fig. 4H; \( P < 0.0001 \)) and all-cause mortality (\( P = 0.0035 \)).

**Multivariate Analysis of Melanoma-Specific Survival**

By univariate Cox regression, the square root–transformed MVD based on antibodies to the CD34 epitope had a quantitatively similar relationship with melanoma-specific mortality than MVD based on FVIII-RAg (Table 3; \( \chi^2 = 19.2 \) versus 17.7; \( P = 0.22 \)). Presence of microvascular loops and networks, presence of epithelioid cells, ciliary body involvement, largest basal tumor diameter, and tumor height also showed a statistically significant association with an increased risk of melanoma-related death in this data set (Table 3). MVD based on antibodies to \( \alpha \)-smooth muscle actin showed no statistically significant association with survival (Table 3).

A multivariate Cox regression model previously fitted to this data set\(^{20}\) was adjusted for the effect of MVD (Table 3). The presence of microvascular loops and networks, modeled by assuming networks to be an advanced stage of loops (\( P = 0.011 \); HR 1.51 for each change in category); the presence of epithelioid cells; and the largest basal tumor diameter retained their significance after adjusting for MVD (\( P = 0.0069 \); HR 1.23 for each unit increase in square root–transformed vessel count obtained with antibodies to the CD34 epitope). Involvement of the ciliary body also retained statistical significance, but the observed number of deaths allowed only four variables to be legitimately entered in the model.\(^{25}\) Forward and backward stepwise regression analysis resulted in the same model. In interaction analysis, no statistically significant difference was shown between results of models that included product terms that involved MVD and variables in the main model and results of the main model.

For comparative purposes, we also fitted the multivariate model developed by Foss et al.,\(^{7}\) which included square root–transformed MVD, evaluated by antibodies to FVIII-RAg, and largest basal tumor diameter (Table 3).

**Discussion**

The present population-based analysis of 167 patients with choroidal and ciliary body melanoma found a strong association between poor survival and high MVD among 134 evaluable patients.\(^{7,8}\) The difference in melanoma-specific 10-year probability of survival was 0.57 between quartiles with the lowest and highest MVD, when globally highest microvessel count was determined with antibodies to the CD34 epitope of endothelial cells. The results were robust, in that basing the analysis on the mean of the three highest microvessel counts, antibodies to FVIII-RAg, and all-cause mortality did not abolish the highly statistically significant association between MVD and death caused by melanoma.

We found strong agreement between the mean of three highest counts and the globally highest vessel count, and no obvious advantage was obtained from counting three areas, as noted by Foss et al.\(^{8}\) The intraobserver reproducibility was satisfactory. Other investigators have reported similar intraobserver and interobserver reproducibility for repeated counts, especially when based on hot spots.\(^{2,9,10}\) It has been found that the hot-spot method is less sensitive to variations within a block, and as a rule one section needs to be scanned to find a representative hot spot.\(^{2}\) We found sections labeled with antibodies to the CD34 epitope more convenient to count than those labeled for FVIII-RAg, and the former method yielded...
higher counts. For these reasons, it seems reasonable to use the globally highest count obtained with antibodies to the CD34 epitope as the default method in further studies. It should be realized, however, that MVD as defined here is only a rough index of relative vascularization rather than an exact measure of the number of microvessels present. It is also possible that not all microvessels are immunolabeled with the antibodies used, and positive immunostaining of cell types other than endothelial cells always remains a possibility.

Our results are in excellent agreement with an analysis recently conducted by Foss et al., 7,8 who found a corresponding difference of 0.58 in 9-year probability of survival among 116 patients with choroidal and ciliary body melanoma, by use of antibodies to FVIII-RAg. In both studies, the risk of death increased from quartile to quartile, and HRs in an identically fitted multivariate model were comparable (1.36 versus 1.44).8

Taken together, these two studies strongly suggest that the higher the MVD, the higher the risk of dying of metastatic uveal melanoma.

### Table 1. Microvascular Density According to the Baseline Characteristics of 134 Cases of Uveal Melanoma

<table>
<thead>
<tr>
<th>Variables</th>
<th>CD34 Epitope</th>
<th>Factor VIII-Related Antigen</th>
<th>α-Smooth Muscle Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36 (7–104)</td>
<td>28 (6–88)</td>
<td>5 (2–24)</td>
</tr>
<tr>
<td>Female</td>
<td>43 (5–121)</td>
<td>35 (8–102)</td>
<td>6 (1–21)</td>
</tr>
<tr>
<td>Tumor location</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Choroid only</td>
<td>37 (5–107)</td>
<td>30 (6–102)</td>
<td>6 (1–24)</td>
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<tr>
<td>Ciliary body involved</td>
<td>44 (11–121)</td>
<td>36 (10–83)</td>
<td>5 (1–13)</td>
</tr>
<tr>
<td>Cell type</td>
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<td></td>
<td></td>
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<tr>
<td>Spindle</td>
<td>34 (5–107)</td>
<td>27 (6–102)</td>
<td>6 (1–24)</td>
</tr>
<tr>
<td>Nonspindle</td>
<td>45 (13–121)</td>
<td>40 (9–83)</td>
<td>5 (1–16)</td>
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<td>Microvascular patterns</td>
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<td></td>
<td></td>
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<tr>
<td>No loops</td>
<td>28 (5–101)</td>
<td>24 (7–102)</td>
<td>6 (2–24)</td>
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<tr>
<td>Loops only</td>
<td>42 (14–121)</td>
<td>27 (6–83)</td>
<td>5 (1–17)</td>
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<tr>
<td>Networks</td>
<td>50 (18–107)</td>
<td>46 (10–88)</td>
<td>5 (1–21)</td>
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<td>Largest basal tumor diameter</td>
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<td>≤10 mm</td>
<td>28 (5–93)</td>
<td>27 (6–69)</td>
<td>5 (1–24)</td>
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<tr>
<td>&gt;10–15 mm</td>
<td>45 (12–121)</td>
<td>37 (8–102)</td>
<td>6 (1–21)</td>
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<td>&gt;15 mm</td>
<td>43 (7–107)</td>
<td>35 (7–83)</td>
<td>5 (2–17)</td>
</tr>
<tr>
<td>Extraocular extension</td>
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<td>No</td>
<td>39 (5–121)</td>
<td>31 (6–102)</td>
<td>6 (1–24)</td>
</tr>
<tr>
<td>Yes</td>
<td>42 (12–104)</td>
<td>45 (10–67)</td>
<td>6 (2–16)</td>
</tr>
</tbody>
</table>

Data show the globally highest MVD, evaluated by the three antibodies. Data are expressed as median (range) MVD.

* Mann–Whitney test, two-sided; unpaired t-test after square root transformation, two-sided.
† Kruskal–Wallis test, two-sided; one-way analysis of variance after square root transformation, two-sided.
FIGURE 4. Melanoma-specific (A through F, H) and all-cause mortality (G) Kaplan–Meier survival curves (crossbars indicate upper and lower 95% confidence limits, and ticks indicate censored observations). The survival of patients with high MVD was shorter than that of patients with low MVD, whether analyzed by the globally highest (A) and the mean of three highest counts (B) obtained with antibodies to CD34 epitope, or the globally highest (C) and the mean of three highest counts (D) obtained with antibodies to FVIII-RAg. Survival was similar regardless of MVD analyzed by the globally highest (E) and the mean of three highest counts (F) obtained with antibodies to α-smooth muscle actin. Analysis based on all-cause mortality and the globally highest MVD obtained with antibodies to CD34 epitope produced equivalent results (G). The presence of microvascular loops and networks also differentiated patients with good and poor prognosis (H).
melanoma. Interestingly enough, MVD assessed in a similar fashion was also associated with the number of metastases in a recently reported experimental murine model of intraocular melanoma.34

In two other data sets, however, opposite results regarding the prognostic significance of MVD in uveal melanoma were obtained. In a case–control study of 63 tumors, Lane et al.10 counted two hot spots at the tumor’s apex, four within the tumor, and four at the tumor’s base. The median MVD ranged from 5.7 to 11.4 vessels per counted area. Patients with metastases had neither a higher total count nor a higher count at any level within the tumor than patients without metastases. In a cohort study of 40 uveal melanomas, Schaling et al.9 determined MVD from five randomly chosen areas. The mean MVD ranged from 1 to 12.5 vessels/0.216 mm² and was not associated with survival. The small number of patients in these two studies may have contributed to the negative results.

The main difference between these studies on MVD in addition to small study sizes, and the most likely reason for different results, is the strategy of selecting areas to be counted. Whereas in the present study and in the series of Foss et al.7,8 the analysis was based on the globally highest MVD, the

### Table 2

#### Ten-Year Cumulative Probability of Survival According to MVD among 134 Cases of Malignant Choroidal and Ciliary Body Melanoma

<table>
<thead>
<tr>
<th>CD34 Epitope</th>
<th>P</th>
<th>Factor VIII-Related Antigen</th>
<th>P</th>
<th>α-Smooth Muscle Actin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma-specific</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>1</td>
<td>0.91/0.87*</td>
<td>0.91/0.90</td>
<td>0.62/0.57</td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>0.71/0.75</td>
<td>0.62/0.67</td>
<td>0.48/0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.41/0.36</td>
<td>0.45/0.47</td>
<td>0.75/0.60</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>0.34/0.42</td>
<td>0.0001/0.0001†</td>
<td>0.61/0.61†</td>
<td></td>
<td></td>
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<tr>
<td>All-cause</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>0.68/0.67</td>
<td>0.71/0.75</td>
<td>0.48/0.40</td>
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</tr>
<tr>
<td>2</td>
<td>0.55/0.53</td>
<td>0.44/0.44</td>
<td>0.39/0.46</td>
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</tr>
<tr>
<td>3</td>
<td>0.33/0.33</td>
<td>0.31/0.37</td>
<td>0.48/0.45</td>
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<tr>
<td>4</td>
<td>0.24/0.27</td>
<td>0.0022/0.014†</td>
<td>0.35/0.27</td>
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</table>

* Data show survival ratio based on single globally highest MVD vessel count/mean of three highest vessels counts, by quartile, evaluated by three antibodies.
† Long-rank test, two-sided.

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In two other data sets, however, opposite results regarding the prognostic significance of MVD in uveal melanoma were obtained. In a case–control study of 63 tumors, Lane et al.10 counted two hot spots at the tumor’s apex, four within the tumor, and four at the tumor’s base. The median MVD ranged from 5.7 to 11.4 vessels per counted area. Patients with metastases had neither a higher total count nor a higher count at any level within the tumor than patients without metastases. In a cohort study of 40 uveal melanomas, Schaling et al.9 determined MVD from five randomly chosen areas. The mean MVD ranged from 1 to 12.5 vessels/0.216 mm² and was not associated with survival. The small number of patients in these two studies may have contributed to the negative results.

The main difference between these studies on MVD in addition to small study sizes, and the most likely reason for different results, is the strategy of selecting areas to be counted. Whereas in the present study and in the series of Foss et al.7,8 the analysis was based on the globally highest MVD, the

### Table 3

#### Univariate and Multivariate Cox Regression Analysis of Survival of 134 Patients with Malignant Choroidal and Ciliary Body Melanoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
<th>Final main model</th>
<th>Model of Foss et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression Coefficient*</td>
<td>Likelihood Ratio†</td>
<td>P</td>
<td>Hazard Ratio‡</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.027 ± 0.010</td>
<td>7.3</td>
<td>0.007</td>
<td>1.03 (1.01–1.05)</td>
</tr>
<tr>
<td>Epithelioid cells§</td>
<td>1.210 ± 0.265</td>
<td>20.7</td>
<td>0.0001</td>
<td>3.35 (2.00–5.62)</td>
</tr>
<tr>
<td>Ciliary body involvement§</td>
<td>0.931 ± 0.269</td>
<td>10.9</td>
<td>0.001</td>
<td>2.54 (1.47–2.57)</td>
</tr>
<tr>
<td>Largest basal tumor diameter</td>
<td>0.129 ± 0.034</td>
<td>13.6</td>
<td>0.0002</td>
<td>1.14 (1.06–1.22)</td>
</tr>
<tr>
<td>Tumor height</td>
<td>0.120 ± 0.037</td>
<td>10.0</td>
<td>0.0016</td>
<td>1.15 (1.05–1.21)</td>
</tr>
<tr>
<td>Extraocular extension§</td>
<td>0.876 ± 0.432</td>
<td>3.3</td>
<td>0.070</td>
<td>2.40 (1.03–5.60)</td>
</tr>
<tr>
<td>Microvascular patterns¶</td>
<td>0.731 ± 0.160</td>
<td>22.8</td>
<td>0.0001</td>
<td>2.08 (1.52–2.84)</td>
</tr>
<tr>
<td>MVD, CD34 epitope¶</td>
<td>0.306 ± 0.070</td>
<td>19.2</td>
<td>0.0001</td>
<td>1.36 (1.18–1.56)</td>
</tr>
<tr>
<td>MVD, FVIII-RAg¶</td>
<td>0.314 ± 0.074</td>
<td>17.7</td>
<td>0.0001</td>
<td>1.37 (1.18–1.58)</td>
</tr>
<tr>
<td>MVD α-Smooth muscle actin¶</td>
<td>−0.081 ± 0.187</td>
<td>0.2</td>
<td>0.67</td>
<td>0.92 (0.64–1.33)</td>
</tr>
</tbody>
</table>

* Regression coefficient ± SE.
† χ² test, two-sided.
‡ Hazard ratio (95% confidence interval).
§ Categories: No, 0; yes, 1.
¶ Categories: No loops, 0; loops without networks, 1; networks, 2.
¶ Square root-transformed single globally highest vessel count.
microvascular densities in the two other studies were probably diluted by counting vessels from predetermined or random areas of the tumor, making the counts notably lower and less variable than when using hot-spot counting.3,10 Areas of highest MVD are also believed to be related to the process of hematogenous metastasis.2–4 Basing the analysis on hot spots is analogous to grading cell type, mean size of largest nuclei, and loops and networks, which are not evaluated from predetermined or random areas.

For technical reasons, the area from which microvessel counts were made varied somewhat: 0.313 mm² in the present study, and 0.250 mm² and 0.216 mm² in the series of Foss et al.7,8 and Schaling et al.,9 respectively. Lane et al.10 did not mention the field used. It is unlikely that these slight differences had a major impact on the results, but in general the area to be counted should not be too small or too large, to avoid exaggerating or diluting the hot spot.3,5 The method of visualizing microvessels also varied. Whereas our preferred marker was CD34 epitope, Lane et al.10 used *Ulex europaeus* agglutinin I, and the other two groups used VIII-R.Ag.7–9 The latter markers are thought to be less sensitive than the CD34 epitope, especially for microvessels seen in malignant tumors,7,23,27,28 and weak or incomplete staining has been reported also in uveal melanoma.7,9,10 Our analysis showed, however, that this difference in sensitivity did not affect results of survival analysis.

We found a statistically significant association between high MVD and presence of microvascular loops and networks, presence of epithelioid cells, and largest basal tumor diameter, as did Foss et al.7,8 Overlap in MVD between categories was pronounced, however, which may explain why the smaller series of Lane et al.10 and Schaling et al.9 did not confirm these associations. Our results differ from the series of Foss et al.,7,8 who found no association between melanoma-specific survival and microvascular loops, networks, and cell type, having once adjusted for the effect of MVD. They may have interpreted microvascular patterns differently from the original description by Folberg et al.,12,13 however, judging from correspondence between the two groups.39 Similarly, cell type did not enter the multivariate model of Folberg et al.,12 whereas it independently contributed to prognosis in our series, which was more population based.

In conclusion, most studies on microvascular patterns9,12,14–16,19,20,25 and both studies that analyzed globally highest MVD7,8 support the theory that microvessels have a major influence on tumor progression of choroidal and ciliary body melanoma. Our study suggests that both factors independently contribute to prognosis. There is little reason to confront either these two methods of analysis or assessment of cytologic parameters such as cell type, especially because tumor vascularization includes both production of new vessels and remodeling of old ones,2,3 and these are processes to which tumor cells themselves may contribute.

**Acknowledgments**

The authors thank Marjatta Koikkalainen and Pirkko Yliharju for meticulous technical assistance.

**References**


